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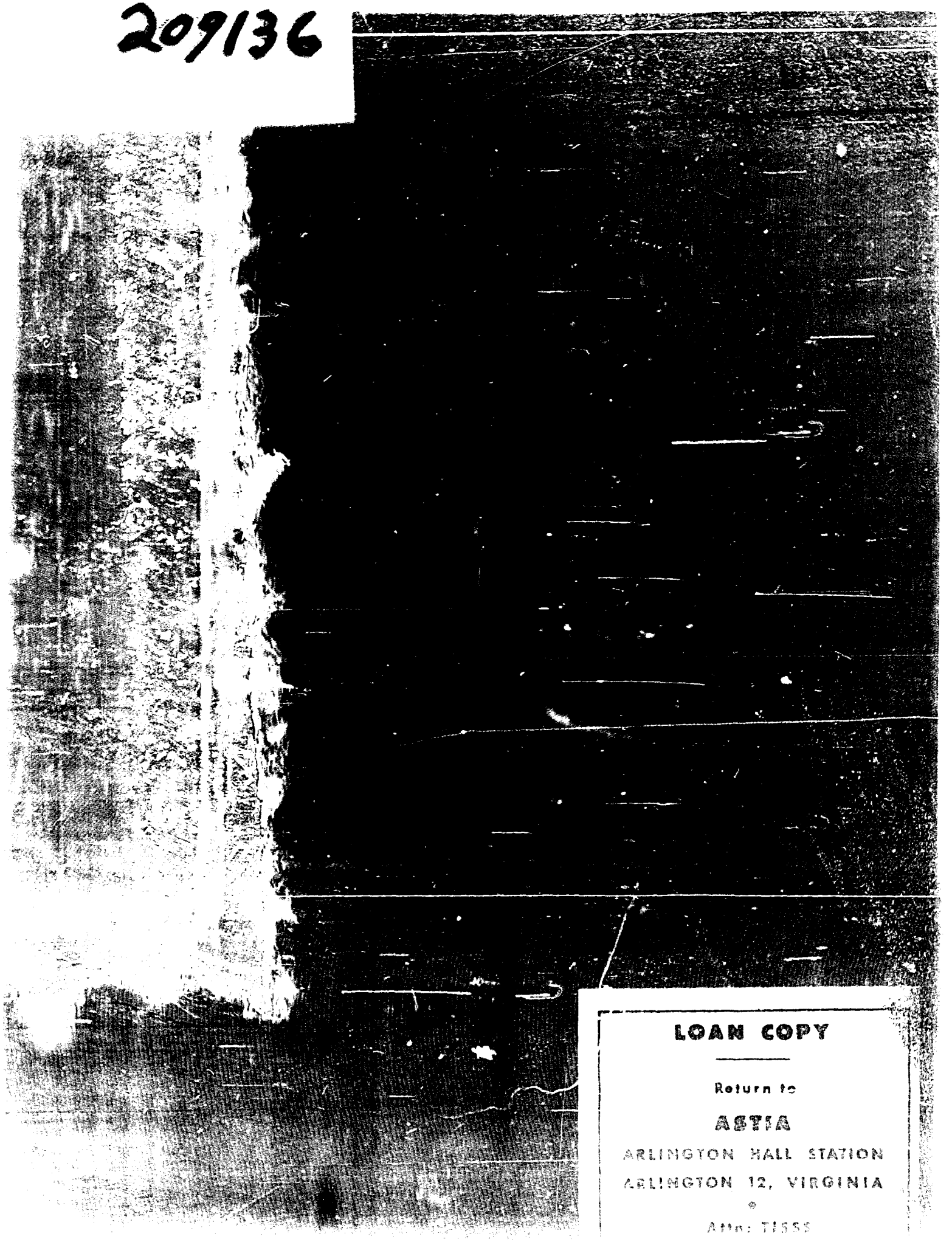

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INTERIM REPORT 182

# PRINCIPLES AND PRACTICE OF BW DECONTAMINATION:

## 25. Third Interlaboratory Conference on Mechanisms and Kinetics of Virus and Spore Inactivation

Edited by

Saul Kaye
and
Robert K. Hoffman

This is a report of record and does not necessarily reflect the doctrine of the Chemical Corps.



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U. S. ARMY CHEMICAL CORPS RESEARCH AND DEVELOPMENT COMMAND
U. S. ARMY BIOLOGICAL WARFARE LABORATORIES
PHYSICAL DEFENSE DIVISION
Fort Detrick, Frederick, Maryland

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## SUMMARY

This report presents an edited, abbreviated version of the proceedings of the Third Interlaboratory Conference on the Mechanisms and Kinetics of Virus and Spore Inactivation held at Llewellyn Farms, Morris Plains, New Jersey, May 6, 1955, and at the Chemists' Club, New York City, May 7, 1955.

# I. INTRODUCTION

In November 1953, June 1954, and May 1955, three successive conferences were held to discuss the subject of the mechanisms and kinetics of virus and spore inactivation. Personnel attending these conferences were engaged in research under contracts with the Decontamination Branch, Physical Defense Division, Fort Detrick. Edited transcriptions of the first two conferences were published as Fort Detrick Interim Reports 67 and 91 and were distributed to various investigators to inform them of the nature and direction of the research of others in closely associated fields.

The proceedings of the third conference are herein similarly collected; however, a reduction in funds for the support of research contracts by Decontamination Branch has since resulted in the cancellation of all contracts on this subject. This report, consequently, may well be the last resulting from the conferences, but we hope it will serve workers in the field of sterilization as a guide in future work.

We have attempted to reproduce herein both the factual data presented and the spirit of informality and cooperation which pervaded the conference. We hope, however, that the conferees will forgive us for taking liberties with their spoken and written words, but we accept full responsibility for any mistaken or incomplete impressions. The participants in the third conference are shown in Figure 1.

This introduction would not be complete without expressing the indebtedness of the Chemical Corps and of all the conferees to Dr. George H. Mangun, Research Director, Warner-Lambert Laboratories, who was host for the conference for the third time. The conference was a success largely because of Dr. Mangun and his staff.

Figure 1. Participants in the Third Interlaboratory Conference. Front row, left to right: R.K. Hoffman, E.C. Mayo, D.M. Portner, S. Yaverbaum, Fort Detrick; M.E. Deutsch, Warner-Lambert Labs. Rear, left to right: G.F Reddish, Warner-Lambert Labs; S. Kaye, Fort Detrick; G. Darker, Ben Venue Labs; B. Schwartz, Warner-Lambert Labs; H.W. Heine, Bucknell Univ.; E. Lasley, Warner-Lambert Labs; R.W. Brockman, Southern Research Inst.; B. Dubnick, Warner-Lambert Labs; B.D. Church, Univ. of Mich.; F.M. Schabel, Jr., S. Res. Inst.; F.S. Engley, Jr., Univ. of Tex.; H.E. Skipper, S. Res. Inst.; G.H. Mangun, Warner-Lambert Labs; H.O. Halvorson, Univ. of Mich.; B. Edwards, Warner-Lambert Labs; F.W. Shaver, B.F. Goodrich Res. Labs; J.R. Leonards, Ben Venue Labs; H.O. Halvorson, Univ. of Ill.; R. Porter, Fort Detrick; R.G. Sanders, Warner-Lambert Labs; T.B. Stin[illegible], Fort Detrick. Conferees not shown in the picture: O.B. Williams, Univ. of Tex.; L.P. Greenberg, Fort Detrick.

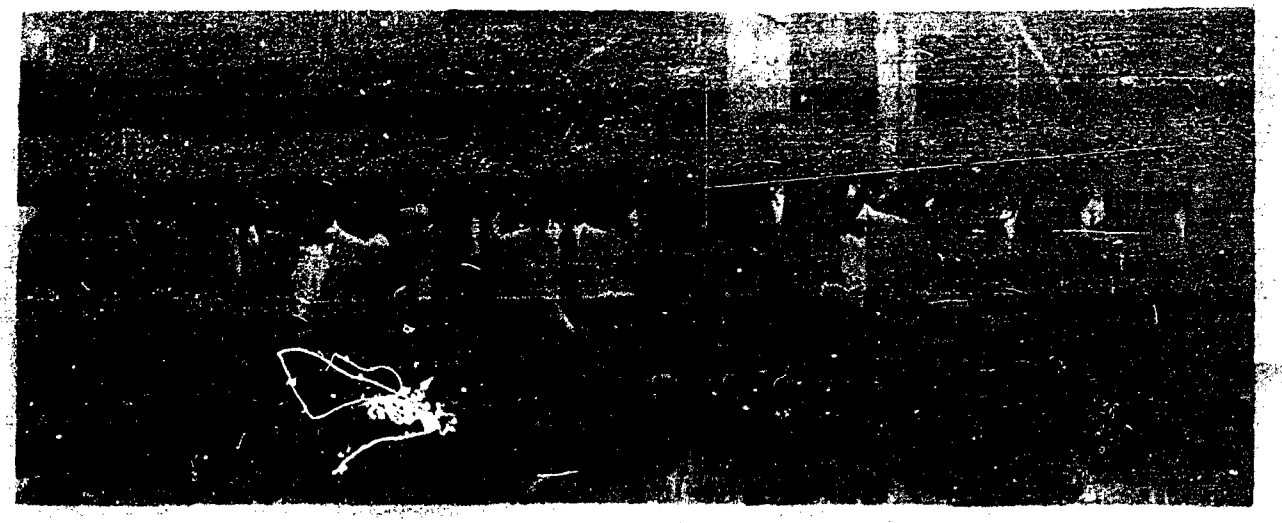


Figure 1. Participants in the Third Interlaboratory Conference. Front row, left to right: R.K. Hoffman, E.C. Mayo, D.M. Portner, S. Yaverbaum, Fort Detrick; M.E. Deutsch, Warner-Lambert Labs. Rear, left to right: G.F Reddish, Warner-Lambert Labs; S. Kaye, Fort Detrick; G. Darker, Ben Venue Labs; B. Schwartz, Warner-Lambert Labs; H.W. Heine, Bucknell Univ.; E. Lasley, Warner-Lambert Labs; R.W. Bruckman, Southern Research Inst.; B. Dubnick, Warner-Lambert Labs; B.D. Church, Univ. of Mich.; F.M. Schabel, Jr., S. Res. Inst.; F.B. Engley, Jr., Univ. of Tex.; H.E. Skipper, S. Res. Inst.; G.H. Mangun, Warner-Lambert Labs; H.O. Halvorson, Univ. of Mich.; B. Edwards, Warner-Lambert Labs; F.W. Shaver, B.F. Goodrich Res. Labs; J.R. Leonards, Ben Venue Labs; H.O. Halvorson, Univ. of Ill.; R. Porter, Fort Detrick; R.G. Sanders, Warner-Lambert Labs; T.B. Stim, Fort Detrick. Conferees not shown in the picture: O.B. Williams, Univ. of Tex.; L.P. Greenberg, Fort Detrick.

## II. MINUTES OF THE FIRST SESSION

**KAYE:** This is the third and last annual gathering of contractors of Fort Detrick who are performing research on the mechanism and kinetics of virus, bacterial, and spore inactivation. All these meetings have been convened by Dr. Mangun, who, in addition to having a contract with Detrick, is coordinator of this phase of our research. It appears desirable to us to have all contractors, ex-contractors, and will-be contractors get together and discuss unclassified work relating to the inactivation of the various types of organisms in which we are particularly interested.

**MANGUN:** I had hoped that Dr. Skipper could be here to act as Chairman for this first session on viruses, but he has been delayed. I think that perhaps the best thing to do under the circumstances is to go ahead with some of our own kinetic studies.

**DEUTSCH:** First, let me review what our thought has been in the past. Our thought has been to get at the mechanism of viral and spore inactivation by studying the kinetics of the inactivations. Our procedure has been very simple: Prepare the suspensions of spores and viruses at a constant pH and temperature, expose them to the inactivating agent, and study the velocity of the disappearance of viability or infectivity.

Making these studies at different concentrations of spore and inactivating agents, we have been able to determine that all of our inactivating reactions are first order with respect to biological agents, and first order with respect to inactivating agents. That is, the mechanism fits a one-to-one reaction, a reaction between one molecule of inactivating agent and one molecule of biological material; by molecule of spore I mean one spore, by molecule of virus, I mean one virus particle. By studying these kinetics at different temperatures, we have been able to derive certain thermodynamic parameters of the inactivating reaction. These have been calculated using the convention that a mole of spores is $6.02 \times 10^{23}$ spores, and a mole of virus is $6.02 \times 10^{23}$ virus particles.

Table I is a summary of thermodynamic parameters that we have determined using this technique. One of these parameters is enthalpy or heat of activation; that is, the change in $\Delta H^{\ddagger}$ occurring during formation of the activated complex, which is an intermediate between the unchanged virucide and the virus and the inactivated virus with the virucide molecule added onto it. The second parameter is the $\Delta S^{\ddagger}$; that is, the entropy change accompanying the formation of the activated complex which is the first stage in the inactivation of the biological agent. You will notice that, although this table treats a number of alkylating agents and a number of biological materials (omitting the first two entries), all of these figures are of the same order of magnitude. The first two rows on this table are a little different from the rest. They are for chemical reaction between propiolactone and alanine. If you study this reaction at a number of different [illegible] find [illegible] velocity [illegible] of the reaction beta

TABLE I. THERMODYNAMIC REACTION PARAMETERS

| Alkylating Agent | Other Reactant | ΔH*, Calories | ΔS*, Cal/degree |
|---|---|---|---|
| Propiolactone | Alanine $-NH_2$ | 36,300 | 64 |
| " | " $-NH_3^+$ | ~13,000 | ~-25 |
| " | Influenza A Virus (pH 6.6) | 11,000[a] | 13[b] |
| " | WEE Virus (pH 6.6) | 17,000 | 5 |
| " | " " (pH 9.6) | 16,000 | 4 |
| " | B subtilis[c] spores (pH 4.6) | 18,000 | 13 |
| " | " " (pH 6.6) | 24,000 | 20 |
| " | " " (pH 9.6) | 20,000 | 17 |
| Ethylene oxide | Influenza A Virus (pH 5.6) | 15,000 | - 9 |
| " | " " " (pH 9.6) | 14,000 | -10 |
| " | WEE Virus (pH 6.6) | 16,000 | - 4 |
| " | " " (pH 9.6) | 16,000 | - 3 |
| Ethylenimine | Influenza A Virus (pH 5.6) | ~18,000 | ~ 9 |
| " | " " " (pH 9.6) | ~16,000 | ~- 1 |
| " | WEE Virus (pH 6.6 and 9.6) | ~20,000 | ~ 10 |
| Dinitrofluorobenzene | " " (pH 6.6) | ~21,000 | ~ 30 |

a. Standard Error = 1000. b. Standard Error = 4. c. var. niger.

propriolactone and alanine is greatly dependent on pH. But, as I will discuss in a short time, we can separate two effects to explain the higher pH variation in the following terms: that the reactions between propriolactone and an ionized or un-ionized carboxyl group is very slow compared to the other reaction; that the reaction with $NH_2$ is rather fast; and that the reaction with $NH_3^+$ is faster than the carboxyl reaction but considerably slower than (maybe one percent of) the reaction with $NH_2$. We can explain the pH variation on that basis. The question naturally comes to mind whether the thermodynamic parameters of the inactivating reactions of the biological materials are compatible with a mechanism in which amino groups are hit. After studying these data very carefully, I come to the conclusion that the two things are compatible, that at any of the pH's at which we studied these biological reactions, the predominating form is $NH_3^+$. You will see that I have tabulated biological inactivating reactions at pH's from 4.6 to 9.6. The values for ΔH* and ΔS* for the inactivation reactions fall within the range that might be expected if amino groups are attacked. That, of course, is not proof that amino groups are attacked, but merely shows that the kinetic data are not inconsistent with those mechanisms.

The values obtained for other protein denaturations that have been determined experimentally range from 40,000 to 140,000, well above the range observed here, and the ΔS* values range from 40 to 300, higher than any of the fi[illegible] amino gr[illegible]

KAYE: Is that thermal denaturation of protein?

DEUTSCH: Thermal denaturation is causing it. However, it has been shown that thermal denaturation, and acid or alkaline denaturation are just different ways of saying the same thing. What it means is that thermal denaturation is much accelerated by a high concentration of hydrogen ions or hydroxyl ions. The processes seem to be essentially the same.

HALVORSON (Ill.): Those are $\Delta H$'s on soluble protein, is that correct?

DEUTSCH: The values that I quoted - 40,000 to 140,000? Yes, they are.

HALVORSON (Ill.): I wonder if it would be valid to compare that to either your spore or your virus system, where your primary effect might presumably be a surface one? Like on receptor sites, or in your virus, some surface protein. Would you expect that to differ very much? Is there any literature on denaturation of structural protein?

DEUTSCH: There couldn't be any literature on the denaturation of insoluble proteins, because denaturation is usually defined as a failure to be soluble at a pH and concentration at which solubility usually occurs.

HALVORSON (Mich.): I realize that, yes, but hasn't it also been used excessively to measure sulfhydryl appearance and disappearance paralleling denaturation? There is a fairly close correlation, I believe, and we wonder if one couldn't do this with a structural protein.

DEUTSCH: I don't know.

KAYE: There is another thought that occurred to me: the protein denaturations for which thermodynamic parameters have been obtained are all reversible in nature. How does this compare with inactivation of organisms?

DEUTSCH: Well, some people say that denaturation is a reversible step followed by an irreversible step. There is no unanimity in the definition of denaturation.

HEINE: How did you measure the rate of reaction of propiolactone and alanine to obtain these heats and entropies of activation?

DEUTSCH: I didn't want to go too much into the technical details, but for measuring the rate of reaction between propiolactone and alanine, we made a solution of alanine at a high enough concentration so that even when all the propiolactone was gone, the concentration of alanine had practically remained unchanged. We added propiolactone to this at a constant known pH and constant temperature and intermittently determined propiolactone concentration by using hydroxylamine and ferric chloride to test for propiolactone.

HEINE: I wonder if you would get your heat of activation of propiolactone and alanine more in line with your viruses and spores here if you had used some dipeptides and tripeptides, rather than the amino acid. You see, you had 36,000 calories involved there to 11,000 to 17,000 calories for the virus. In amino acids, you have a highly polar group. You cut down that figure when you get into the dipeptide and tripeptide and a tetrapeptide, and I think the entropies and heats of activation would be more in line with your spores and viruses. After all, you are dealing with a polypeptide there in viruses and spores.

DEUTSCH: Those studies certainly are indicated and are planned. Please remember that the $NH_2$ group reaction is not the predominating one, but the $NH_3^+$ group reaction predominates at the pH's we studied, which incidentally are the only practical pH's, since we get into technical difficulty above or below the pH's used. Your suggestion is a very good one, and this is just the first step in our study of chemicals to compare with the actual biological system.

SHAVER: Do you think if you used mercapto acetic acid instead of alanine, it would make much difference in your energy? The mercaptones are rather inactive toward the lactone until it is at a high pH and then it is very rapid.

DEUTSCH: We have experience with glutathione, and this gets too fast to measure. We couldn't study it that way because the mercapto acetic would interfere with the hydroxylamine reaction because of a complex with the ferric ion.

In previous studies that we reported at other meetings with Western equine encephalomyelitis virus, the virus was assayed in every case by using mice to determine the lethal dose. We thought it would be good to see if our results were more general than that; that is, whether or not we were studying loss of virus activity or whether we are just studying loss of virulence to mice. So we have compared on the next graph (Figure 2) the loss of virus titer as measured by fibroblast and the loss of virus titer as measured by mouse assay. The two follow one another very closely.

STIM: What type of tissue culture assay was that?

DEUTSCH: Essentially the Dulbecco technique.

STIM: I recall at the last meeting, Dr. Schwartz had quite a bit of difficulty in getting successful results with this method. I am quite surprised that you have accomplished it at this time.

DEUTSCH: It is difficult to get it started, but once you do, you can get good results.

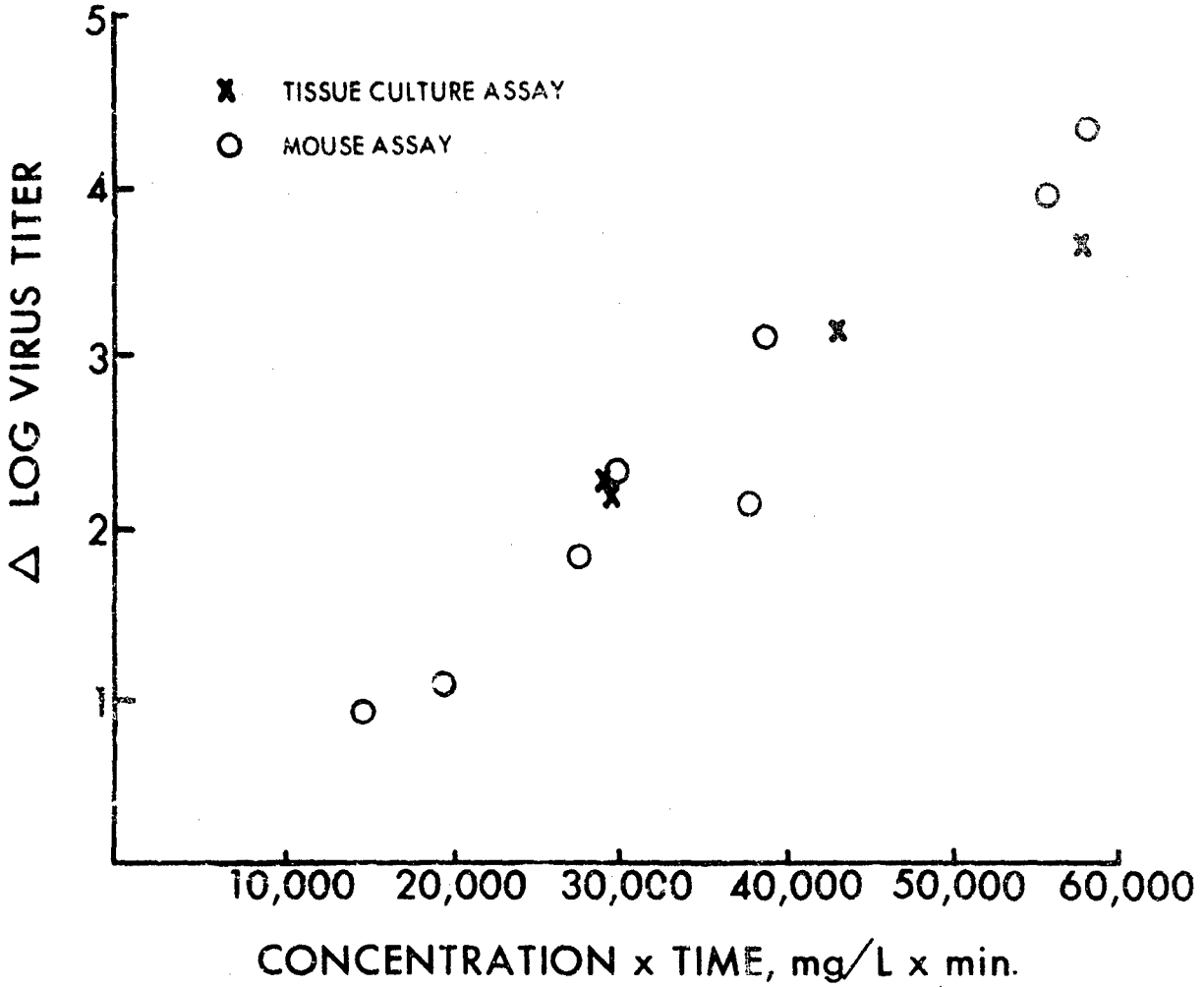



Figure 2. Inactivation of WEE Virus by Beta-propiolactone in pH 6.60 Phosphate Buffer.

**MANGUN:** I might add that we have done a similar experiment in years past with sulfur and nitrogen mustards, and the slopes of the inactivation curves have been the same irrespective of the test system that we used. Where we could use the virus in the egg embryo and the same virus intercerebrally, and perhaps by injection into the paw, the slope of the inactivation curve was the same. The absolute points, of course, changed due to the infectivity of the host. I have taken both these data and Dr. Deutsch's data recorded here to indicate that whatever we are doing to the virus has to do with something at the cellular level. There seems to me to be no doubt but what these alkylating agents are affecting the virus so that it behaves differently at the cellular level, either in the adsorption, or at some subsequent point in the reaction, and not prior to that.

**REDDISH:** Is it possible to determine the numbers more accurately by *in vitro* tests (that is, on the plates) than by *in vivo* tests?

**DEUTSCH:** It seems to take more virus particles to make one plaque that way on a plate than to infect the animal. This strain of virus has been used for a long time on mice, and then we started to measure it by chick fibroblast tissue culture. It took more virus particles to get a plaque than to kill a mouse.

**REDDISH:** It would depend upon which virus you used too, wouldn't it?

**MANGUN:** When you prepare a mass of cells, you undoubtedly get growth of only a small percentage of those cells, whereas the virus is probably absorbed on cells irrespective of whether they are living or dead. In your host system, a higher percentage of cells are viable, and this may be a key factor in the sensitivity or the titer differences.

**DEUTSCH:** We don't know exactly, but I know this, that comparing the two results, either it takes a number of particles to make a plaque, or it takes a fraction of a virus particle to infect a mouse.

**DUBNICK:** In other words, the term "viable" means several things?

**SCHWARTZ:** No, in this case viability means the ability to attach, enter, and reproduce. If any one of these stages fails to occur, you may not have a viable virus. If it doesn't attach, then, of course, it isn't a viable virus. If it doesn't reproduce in the cell, it can't be called a viable virus. And if it isn't released, you won't know whether it is a viable virus. So all those things have to take place before our criterion of viable virus is met.

ENGLEY: You have to be careful of the term sensitivity as opposed to accuracy. We ran into something analogous to this in working with *Pasteurella tularensis* while trying to compare plate counts with infectivity. We found, in one particular instance, that we were getting an $LD_{50}$ of one-third of an organism compared to plate counts, because the plate count was not as sensitive as the infectivity to the animal. I think the important thing is to determine the accuracy of the particular technique.

DEUTSCH: That is an experience comparable to ours.

HALVORSON (Ill.): But still you say it takes so many to produce a plaque. I do not think that is true.

DEUTSCH: I don't mean that they will cooperate in producing the plaque; I mean in order to form a plaque, you must have that number present. Then it is probable that one of them is going to give us a plaque. I don't mean that they all stick together to make a plaque.

MANGUN: As I mentioned before, the obvious interpretation here has to be that the number required is very high, because we are quite certain that in our test systems in the animals the number of particles required is very high. We have all these dynamic operations that are going on to neutralize a large percent of the virus so that we are actually not counting particles as stated, but are counting proportionality, and that is about as far as we could go. We would hope that eventually we would get such fine tissue cells that even a single virus would be capable of reproducing, and thus that each virus particle was capable of infecting. In that case, we would get a one-to-one ratio, but I don't think anyone has approached that in a viral system.

HALVORSON (Mich.): Do you have any information on the direct count of particles by electron microscopy?

MANGUN: No, we do not. But even then the electromicrograph would not show whether the particles were viable.

HALVORSON (Mich.): No, that's true, but it might give you a little closer base line for comparison. I think it has been done with Influenza A. I think it would be important to have as many observations of this multiplicity effect where you can get cooperative effects. If you are going to be dealing with a very large multiplicity effect per cell, then this may influence your kinetic interpretation.

DEUTSCH: Of course, the multiplicity effect applies only to an animal like a mouse. You can't get them with a plate.

HALVORSON (Mich.): Well, you can get this effect with bacteriophage. I don't know whether it has been done with the influenza.

DEUTSCH: We diluted out the virus to the extent where we feel certain that the influence of individual virus particles did not overlap.

HALVORSON (Mich.): If you do not know the number of particles that you are dealing with, how do you know how much to dilute? The multiplicity effects are generally around 8, 10, and 15. If you don't know the number of total particles you have, then you don't know exactly what it means unless you know that one particle will cause infection in the mouse, which is an assumption.

MANGUN: Well, the multiplicity effect actually has to arise in a single cell. The genetic viral units, in the phage work at least, have to be placed in a single host cell in order to effectively put together all the necessary elements for reproduction, and this has to be at the unicellular level.

DEUTSCH: It is very unlikely that this is going to happen because there are thousands of cells on a plate, and to show multiplicity on a plate, you have to have the remarkable coincidence of two virus particles hitting the same cell.

Now, I would like to discuss our studies with alanine. The points on Figure 3 were obtained experimentally. The curve that is drawn there is a theoretical curve worked out from the experimental data. You will notice that under these circumstances the reaction is fairly constant and very slow up to about pH 7, and then starts rising, not in a straight line, but in what looks very much like a titration curve. I would like to tell you how we calculated the velocity of reaction of the amino and ammonia groups from these data. First, at the very low pH's, the concentration of $NH_3^+$ is far greater than the concentration of $NH_2$, so we can assume as a first approximation that the reaction at very low pH's is due solely to the reaction with $NH_3^+$. Taking that as the first assumption, we would then assume that, at any other pH, the measured velocity is merely the sum of two things: the velocity constant due to $NH_3^+$ times the concentration of $NH_3^+$ and the velocity constant due to $NH_2$ times the concentration of $NH_2$. So we can take every measured data and detach from it how much of it is due to $NH_3^+$, because we look it up to get a series of values for K. The same procedure was used successfully at 25°C, as shown in Figure 4.

However, when we studied the velocity of the reaction between alanine and propiolactone at 37°C (Figure 5), we weren't able to fit a curve to the data well using the pK in the literature. The solid line is the curve obtained by using pK in the literature, pK 10.45. We got a much better fit if we repeated the calculations using a pK at 10.00. There is one point I would like to make very clear. At the lower pH's, $K^1$ $NH_3^+$ predominates. At higher pH's there is more $NH_2$ present and the reaction with $NH_2$ predominates. Where $K^1$ $NH_3^+$ predominates, the reaction is very slow and as a matter of fact, the points on this curve are the differences between two small numbers because we, of course, correct our data for the reaction of propiolactone with water and hydroxyl ion. That correction is of the same order of magnitude as the measured velocity at the lower pH's. Therefore, due to the nature of this matter, we cannot obtain $K^1$ $NH_3^+$ with any degree of accuracy. But we can get $K^1$ $NH_2$ very accurately because, under conditions where it predominates, the correction is a small fraction of the observed velocity. The next curve (Figure 6) is for phenylalanine and propiolactone. It is similar to the others. I have given only one curve for phenylalanine and propiolactone because I could not find pK values for phenylalanine at temperatures other than 25°C in the literature. Apparently no one has bothered to find the pK of phenylalanine at any other temperature so, although we have kenetic data, we can't construct curves to fit them except by guessing the pK.

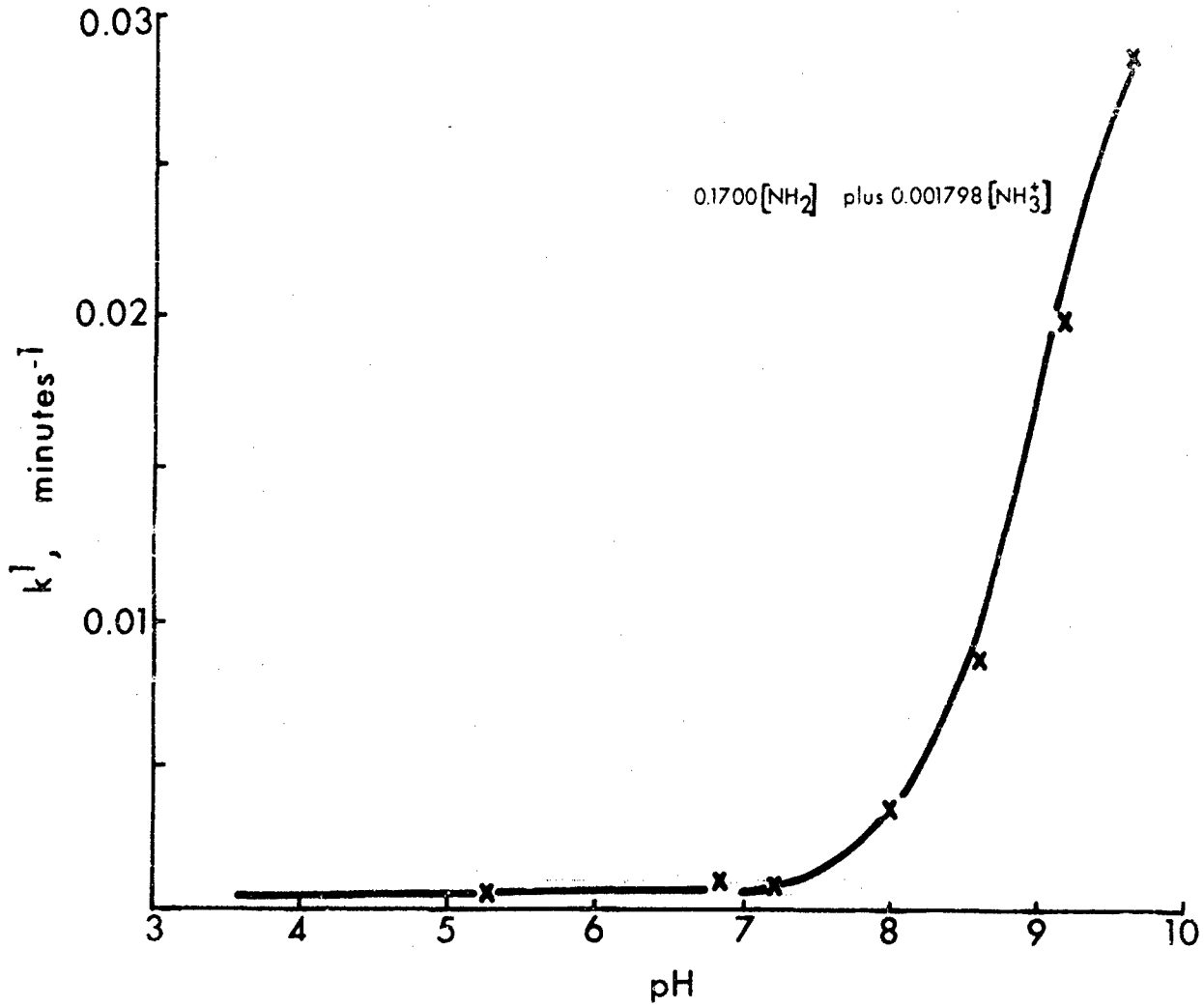



Figure 3. Velocity of Reaction of 0.2 M Alanine with Beta-propiolactone at 15°C.

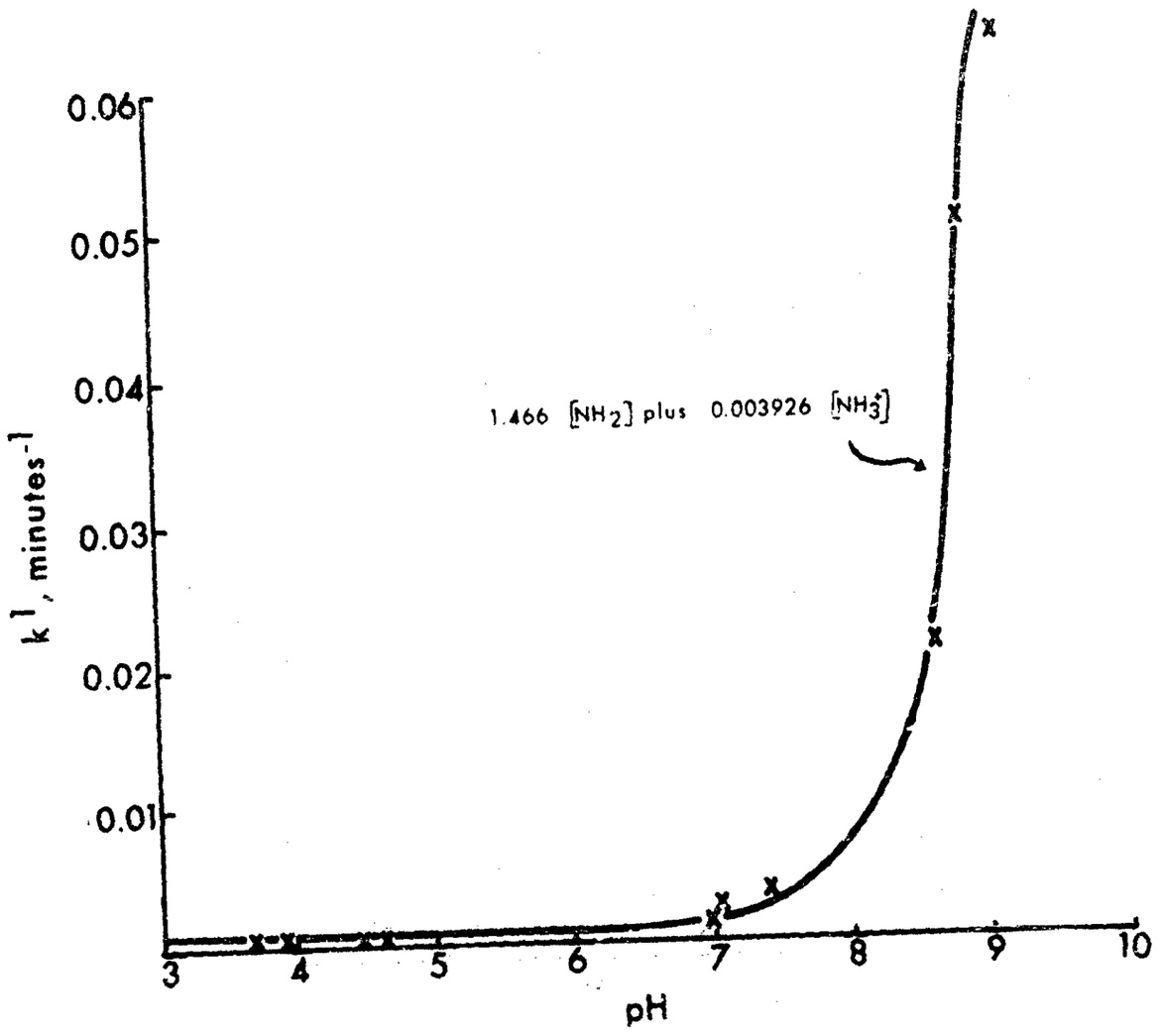



Figure 4. Velocity of Reaction of 0.2 M Alanine with Beta-propiolactone at 25°C.

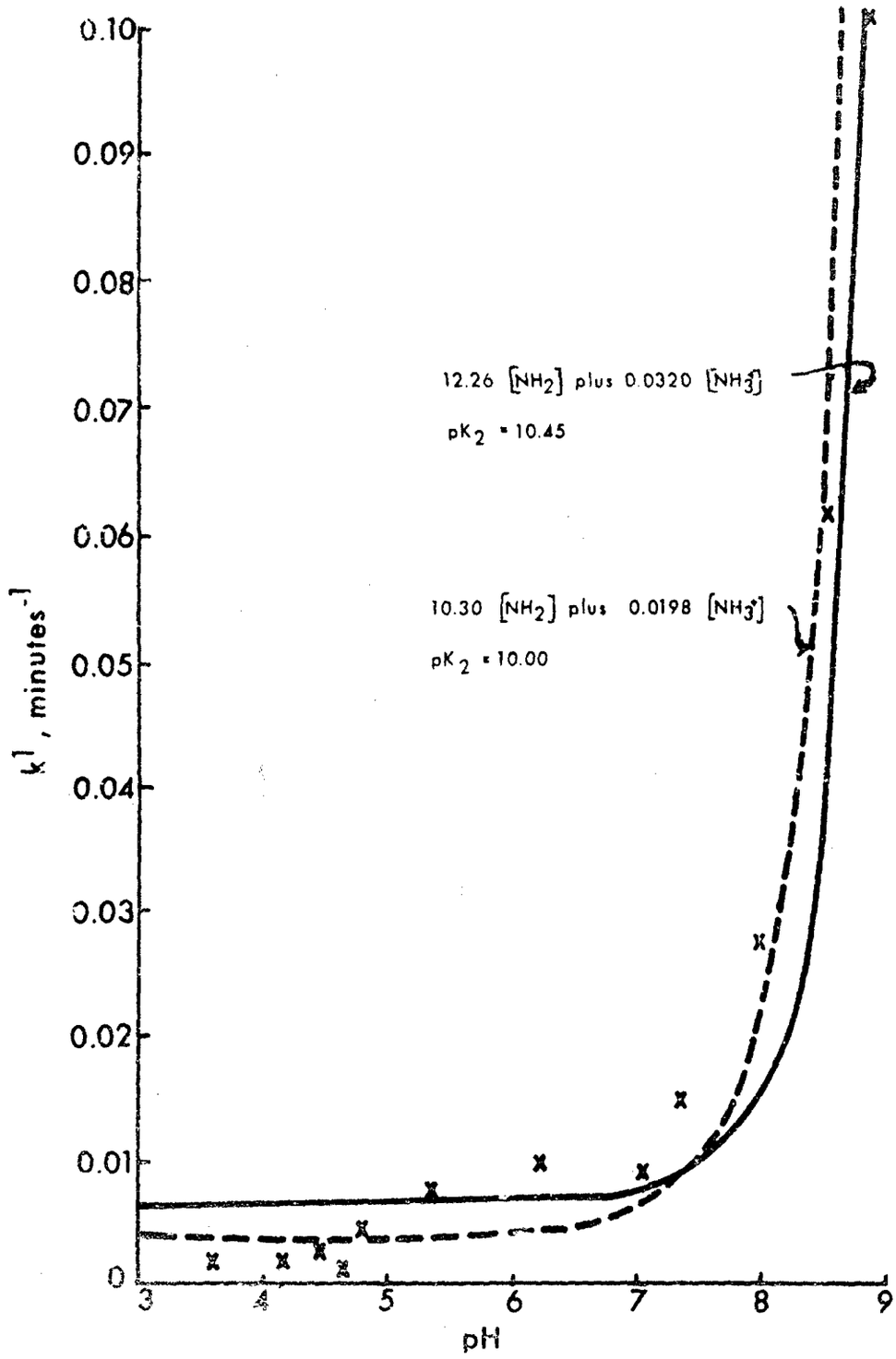



Figure 6. Velocity of reaction of 0.2 M Alanine with β-propiolactone at 37°C.

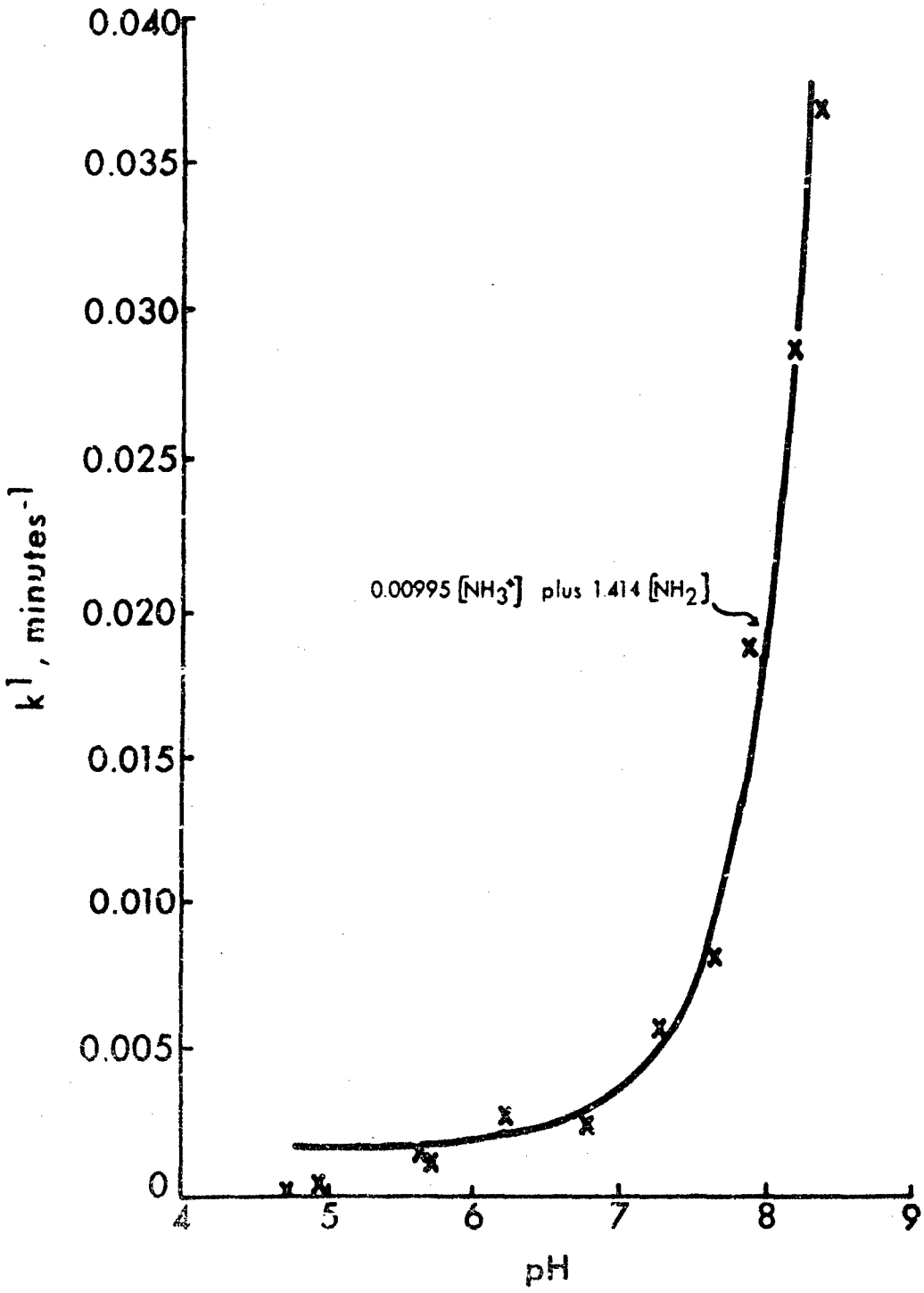



Figure 6. Comparison of Experimental Data with Theoretical Curve for Velocity of Propiolactone Disappearance from Saturated Solutions of Phenylalanine at 25°C.

HEINE: These are pseudo first-order constants, aren't they, because you are holding one reagent in great excess?

DEUTSCH: Yes, there is a constant alanine concentration, so that is why it is K.

HEINE: Couldn't you get the second order constant by dividing by the concentration of the one in excess? After all, you have a reaction going on between the alanine and the lactone. Couldn't you get a second order constant out of that?

DEUTSCH: No, because this $K^1$ is equal to the sum of the concentration of $NH_3^+$ times the velocity constant, and the $K^1$ is equal to the sum of the concentration of $NH_2$. There are two things here, and you can't separate them.

HEINE: I wonder how these K's for the $NH_2$ compare with Bartlett's work with propiolactone and amines, $RNH_2$, where we have the $NH_2$ group alone without having the acid group. Are they comparable?

DEUTSCH: I don't remember the value for the amino group. I'm familiar with Bartlett's studies.

HEINE: You also say that the $NH_3^+$ reaction is very much slower than the $NH_2$ reaction. From the $\Delta H$ and $\Delta S$'s here the free energy of the two reactions are almost the same (different by one kilo calorie) so you wouldn't expect much difference in the reactivity. In the $NH_2$, the entropy is helping the reaction go much faster. This is positive 64 units, while in the $NH_3^+$ the entropy is hindering the reaction even though you have a lower energy of activation (13,000 calories), but $\Delta S$ comes out 19 for the first one and 20 for the second, approximately. You would think, according to the data that you have in the first table, that these two reactions would be going at a comparable speed.

DEUTSCH: All it tells us is that they have the same temperature coefficient.

HEINE: The free energy of activation determines the rate of reaction and that is made up for your two constants.

DEUTSCH: That is at a constant temperature.

HEINE: Over a 10°-, 15°- or 20°-range, there is not going to be much difference. That is not going to affect your $\Delta H$'s.

DEUTSCH: Let us go on to Table II. I told you that we determined these values using statistical techniques, and that enabled us to get the standard deviation of the value. In this table, you will see that we know $K^1$ $NH_3^+$ with a very low order of accuracy, and that, so far as we can tell from our data, the values for the two different kinds of amino groups, charged and uncharged, are not affected by the presence of phenyl groups at the end of the chain.

TABLE II. VELOCITY CONSTANTS FOR REACTION OF PROPIOLACTONE WITH CHARGED AND UNCHARGED AMINO GROUPS AT 25°C

| Molecule Bearing the Group | $k^1_{NH_3^+}$ ± Std. Deviation | $k^1_{NH_2}$ ± Std. Deviation |
|---|---|---|
| Alanine | 0.0039 ± 0.0038 | 1.466 ± 0.004 |
| Phenylalanine | 0.0100 ± 0.0036 | 1.114 ± 0.001 |

I would like next to introduce a concept relating the various rate constants to the practical effectiveness of such compounds as beta-propiolactone.

G. H. Mangun has shown that the effectiveness of a virucide ($\int c\,dt$) is proportional to $(C_o - C)/k_d$, where $C_o$ and C are its initial and final concentrations and $k_d$ is the specific reaction rate of its disappearance from solution.

Let us consider a virucide reacting until its concentration reaches some arbitrary fraction, 1/n of $C_o$. Then

$$\int c\,dt = \frac{(n-1)C_o}{nk_d} . \qquad (1)$$

Suppose we have a virus suspension with an initial $ID_{50}$ of $T_o$. The instantaneous virus titer at any time, t, can be calculated from the expression

$$\text{antilog } T = (\text{antilog } T_o)e - k_i \int c\,dt , \qquad (2)$$

where T is the instantaneous titer and $k_i$ is the velocity constant for virus inactivation.

Substituting (1) in (2), we arrive at

$$\text{antilog } T = (\text{antilog } T_o)e - \frac{k_i}{k_d}\,\frac{(n-1)C_o}{n} \qquad (3)$$

from which it is apparent that the total effectiveness of a virucide is proportional to $k_i/k_d$, if the measure of its total effectiveness is the drop in titer produced when virus is exposed to an initial concentration of virucide, $C_o$, and is left in contact with the virucide to drop to an arbitrary and presumably ineffective level, $C_o/n$.

Let us adopt as a measure of total effectiveness T.E. = $k_i/k_d$. When spontaneous (thermal) inactivation of virus occurs at an appreciable rate, it will have to be corrected for it.

For convenience, let us consider one liter of solution. The argument, however, will apply to any volume.

The velocity constant of virucide disappearance (a pseudo first-order reaction) is (per liter):

$$k_d = \frac{\text{moles virucide reacting with solution constituents}}{\text{virucide concentration x minutes}} . \quad (4)$$

The velocity constant of virus inactivation (a second-order reaction) is:

$$k_i = \frac{\text{virus particles reacting with virucide}}{\text{virus particles per liter x virucide concentration x minutes}} . \quad (5)$$

If one molecule of virucide inactivates a virus particle (which we have always found to be true), the number of virus particles reacting with virucide equals the number of virucide molecules inactivating virus. Let us call this quantity $V_i$.

The number of virucide molecules reacting with solution constituents is N (Avogadro's number) times the number of moles of virucide so reacting. Let us call this product $V_d$. Then from (4),

$$Nk_d = \frac{V_d}{\text{virucide concentration x minutes}} . \quad (6)$$

Now, we defined T.E. as $k_i/k_d$. From (5) and (6),

$$\text{T.E.} = \frac{k_i}{k_d} = \frac{V_i}{V_d} \times \frac{N}{\text{number of virus particles per liter}} . \quad (7)$$

T.E. can thus easily be converted to the ratio of amount of virucide inactivating virus to the amount of virucide being decomposed by other solution constants. To become this ratio, it must be divided by N and multiplied by the number of virus particles per liter. If one virus particle constitutes an infectious unit, this latter figure is 0.693 antilog $ID_{50}$. At any rate, for a given set of starting conditions, T.E. is proportional to the effectiveness ratio described above. Table III contains total effectiveness data for beta-propiolactone for both the influenza and WEE viruses.

You will notice that sometimes we get two figures for the same thing, and that is because we measured it twice and didn't get the same answer both times. The first place this occurs is for influenza A reacting at pH 6.60 in phosphate buffer at 15°C. The figures were obtained with two different pools of influenza virus, so I don't think the agreement is too bad. The second time this occurs is with Western equine encephalomyelitis, which I am sure all of you realize is abbreviated WEE. There are two figures for the rate of reaction of propiolactone with solution constituents (Kd),

TABLE III. TOTAL EFFECTIVENESS DATA FOR PROPIOLACTONE

| Virus | pH | Buffer | Temp. | k | $k_d$ | T. E. |
|---|---|---|---|---|---|---|
| Influenza A | 5.64 | acetate | 15°C | 22.6 | 0.000575 | 39,300 |
| | 6.60 | phosphate | 15° | 26.7 | 0.00169 | 15,800 |
| | | | | 27.7 | | 16,400 |
| | | | 25° | 38.9 | 0.00637 | 6,110 |
| | | | 37° | 116.1 | 0.01983 | 5,860 |
| | 7.54 | | 15° | 29.4 | 0.00479 | 6,140 |
| WEE | 6.60 | | 15° | 11.9 | 0.00228 | 5,220 |
| | | | 30° | 54.2 | 0.0125 | 4,340 |
| | 7.54 | | 15° | 13.0 | 0.00309 | 4,200 |
| | | | | | 0.00404 | 3,220 |
| | | | 22.5° | 25.9 | 0.00710 | 3,640 |
| | | | 30° | 49.0 | 0.0153 | 3,200 |
| | 8.55 | borate | 15° | 12.8 | 0.00144 | 9,000 |
| | | | 30° | 46.7 | 0.00847 | 5,520 |
| | 9.60 | | 15° | 15.8 | 0.00427 | 3,720 |
| | | | | | 0.00330 | 4,820 |
| | | | 30° | 68.5 | 0.0176 | 3,890 |

which might indicate that we have two figures for a pure chemical reaction, but it is not a pure chemical reaction. The solution constituents include pieces of mousebrain homogenate and the variability due to the different reactions of mousebrain homogenate. I believe that there is some evidence that mousebrain homogenate catalizes the disappearance of propiolactone; so we have two figures, and the total effectiveness is calculated from both. You can come to two or three conclusions from this table: low pH favors greater total effectiveness, low temperature favors greater total effectiveness and the buffer has an effect. For a given buffer, low pH and low temperatures are desirable to increase total effectiveness. However, it is nice to use a buffer such as borate, which doesn't react with your alkylating agent, rather than one like phosphate, that does.

REDDISH: This is on virus of influenza A; a lot of work has been done with the Melbourne strain. I wondered whether you have made any comparative tests with the Melbourne strain?

DEUTSCH: No, these are all PR8 strains. There is another bit of information that I would like to add now. We did one experiment to test the total effectiveness theory. We actually determined total effectiveness by using low concentrations of virucide. Within a reasonable time the virucide was all gone, and we determined its total effectiveness. There was quite a bit of variability in the experiments, but the results were consistent with this being the correct hypothesis.

MANGUN: This particular work was done for two reasons. First of all, it is important to any application of this particular agent to know how much it takes to do a certain job. This is important in any possible future uses that the Detrick group might want. Another reason why I was intensely interested in it was a discussion I got into with some very competent virologists about whether to use a low pH or high pH, and high temperature or low temperature. In general, the virologists who were doing the work were very much impressed by the velocity of the reaction and relatively unimpressed by the end results, particularly because the most valid test system they had best measures the short term high velocity reaction. In applying this to human plasma in the inactivation of homologous serum hepatitis, no one would adhere to my idea to keep the pH and the temperature low, and yet I was sure I was right, from fairly authoritative experiments. The present experiments are sufficiently accurate to prove the point, and there is no doubt about it anymore. It was very difficult to convince anyone, however, until these data were really tied down. As a result, some of the work of others on human infections with homologous serum hepatitis were carried out under the least favorable conditions for getting inactivation of the virus. Those that I personally carried out, in which I stuck to the thesis of low pH and low temperature, all worked fine, and the subsequent work may or may not have been seriously different from what would have been obtained if it had been repeated in the original fashion.

REDDISH: The same is true of chlorine compounds. In the alkaline range, they don't work well until we get down to pH 7, and then they begin to be much more active.

MANGUN: Yes, it certainly is pH dependent, as are so many other inhibitors.

DUBNICK: I would like to mention the importance of the rate of disappearance of the virucide as pointed out by experiments with ethylenimine. The rate of inactivation of the virus is very much slower with ethylenimine than it is with propiolactone. However, the total effectiveness of ethylenimine is perhaps twice as great as the total effectiveness of the lactone.

LEONARDS: There is one point I keep insisting on getting on the record every year; this is the third conference, and it is the third time I have made this point. I am sure the question arises in many minds as well as in mine. I think the data you have obtained here are really good, and that it is really important to know exactly how fast and how effective the virucide is under various conditions of pH, temperatures, time, concentration, etc. I still fail to see how the kinetics of the reaction calculated in any manner whatsoever leads you to any understanding of the mechanism by which that inactivation has taken place, especially as a result of all the variables in force. I want to point out again, just for the record, that in the past, any efforts that have been made to get at the mechanism of a biological reaction by kinetic data has led to almost complete disaster in most cases. Now, I know the question is in many people's minds at this time. I fail to see what we have gained by these kinetics, other than the useful data which you can use for practical purposes.

DEUTSCH: We can eliminate certain mechanisms by kinetics. Certain mechanisms are incompatible with the kinetically derived data.

LEONARDS: Well, I will go along with that.

DEUTSCH: Sometimes you have to supply yourself with indirect evidence for seeing whether mechanisms would seem reasonable. I admit that you can't say that because of the kinetics, the mechanism is so-and-so. You can say that because of the kinetics, the mechanism cannot be so-and-so.

SKIPPER: Why did you pick on alanine?

MANGUN: This was set up as the first of a series of similar studies on other reactive groups. It was a matter of getting at one of the most frequently recurring groups on the surface of proteins, to gain some understanding of the facts.

SKIPPER: The only point I would make is that in the work with mustard, which may or may not be appropriate to this discussion, it takes so much mustard to inactivate an enzyme system in vitro, that it almost rules out this type of reaction with proteins. I mean the fact that it takes something in the order of about a 1/10 molar solution of mustard to inactivate most enzyme systems that have been studied, whereas the concentrations that have caused profound effects on cell division and even death in animals, are a thousandfold less than that.

DEUTSCH: We are interested in microorganisms.

SKIPPER: Oh yes, I am fully aware of that, but I assume there are no clinicians among us, and I assume the group feels that one biological system isn't too much different from others.

DEUTSCH: Well, you can't say in our set of circumstances that one concentration is effective and another isn't. It is just that one concentration takes a certain time, and another takes a longer time.

SKIPPER: I agree. However, some of the activity of compounds like the mustards would suggest to me that we aren't dealing with reaction of an alkylating agent on amino groups.

MANGUN: I, myself, am convinced that beta-propiolactone is acting in a manner analogous to that of the mustards. Dr. Deutsch and I have discussed this in the past, and I only agreed to it after the argument that this was a study of amino groups. This could be a study of an amino group substituted on any one of a hundred different systems, including the purines, pyrimidines, and other related compounds. I think that reflects our own feelings on it. I feel, as you do, that there is very little doubt that these alkylating agents are getting into the nucleic acid system, as some of your work has shown.

SKIPPER: Well, another thing that has convinced me is the fact that such extremely small amounts of alkylating agents like triethylene melamine, and nitrogen mustards, will inactivate a biological system of the transforming principle type at about $10^{-4}$ molar in five minutes. No other type of agent or antimetabolites will do this at all, and here you have another active biological material which gives a compatible observation at low concentration.

DEUTSCH: The reason we started with alanine was that it is the easiest one to study. It is soluble and easy to get in high concentration. By no means do we intend to stop here, and by no means would we draw conclusions from this very limited study, but we feel that a study of the amino acid is necessary in addition to the studies of the purines and pyrimidines.

SKIPPER: Oh yes, I certainly agree with that. I think your studies on the amino group compared to the ammonia type are very, very interesting. I just wondered and want to get cleared up in my mind your feeling about the primary biological site.

DEUTSCH: We don't know.

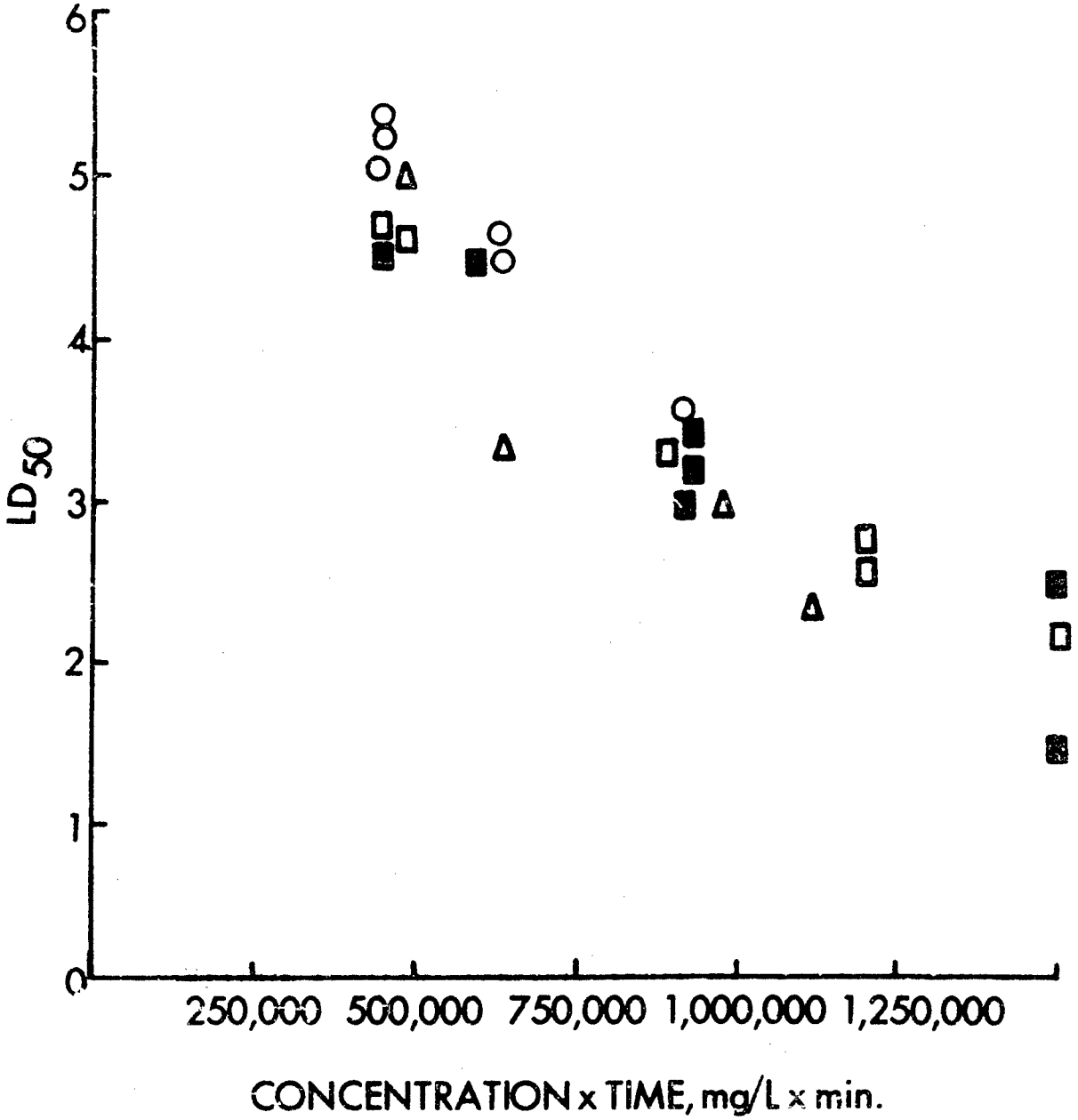
HALVORSON (Mich.): If I might put in a point here, I agree with Skipper on the importance of the nucleic acid. We have been following that for some time, too. But I would like to point out, first, that you are dealing with a system that has to germinate, which requires something special that is not involved in the nucleic acid metabolism as near as is known from the work of Fitzjames and others. Second, you are involved with a system which must primarily be absorbed on a receptor cell, and you know that the ghost cells can absorb even without nucleic acid. So you have here the possibility, in both of these test systems you are using, of key fundamental primary steps which appear not to involve nucleic acid. I think that one should not be overwhelmed then by the nucleic acid data in dividing biological cells. There is quite a difference, first, between the growth of E coli and the germination of the spore and, second, in the absorption of virus. Biologically, they are fundamentally different. You have the possibility of some other receptive center which is specific, maybe sensitive as certain specific enzymes are, to particularly low concentrations of inhibitors, and I think one should have a pretty broad viewpoint at this time.

SKIPPER: I think anyone should have a broad viewpoint, but you are going into matters here of about five years and about $10,000,000 worth of research on the nitrogen mustards when you make a statement like that.

HALVORSON (Mich): That may be very true, but there is enough evidence here that nucleic acids are not involved in the early steps which may be sensitive here, points which may be checked without $5,000,000 worth of research.

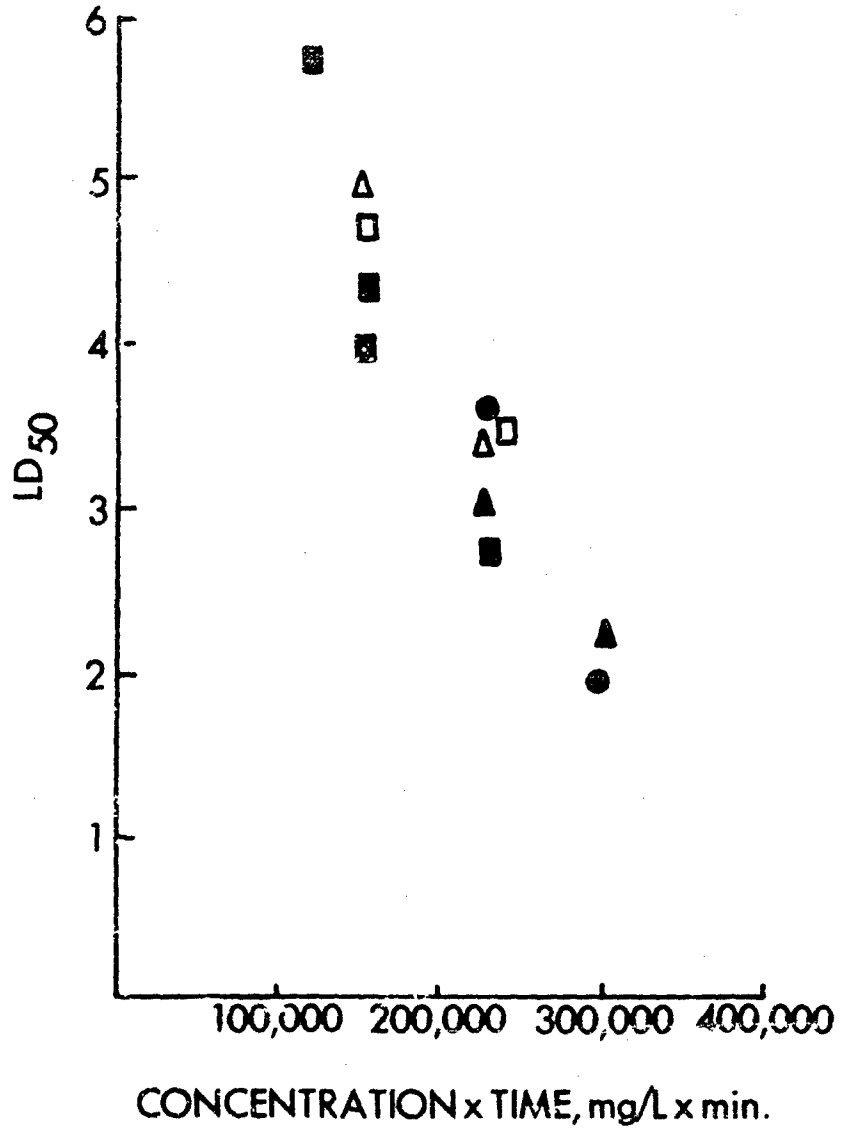
**DUBNICK:** At the last meeting, and again today, we emphasized the importance of analyzing our data statistically. At the last meeting, I was of the opinion that our kinetic results on the inactivation of WEE virus with ethylene oxide indicated no effect of pH; however, in Figure 7, it is apparent that there is a significant difference in the reaction rate between the reaction carried out at pH 6.6 and that carried out at pH 9.6. Again in Figure 8, showing inactivation of WEE virus by ethylene oxide at 30°C, there is a statistical significance in the difference between the reaction at pH 6.6 and at pH 9.6. The rate of this reaction increases with increase in pH. Also the reaction is, as in the case of beta-propiolactone, second order; that is, first order with respect to virucide and first order with respect to virus. Several months ago we studied the inactivation of WEE virus with ethylenimine , and we detected a great deal of variability within the ethylenimine, depending on the source of the virucide and on the pool of virus which were used. In Figure 9, it is apparent that ethylenimine obtained from the various sources and ethylenimine used on different pools of virus gave divergent results. However, with one source of ethylenimine and one source of the virus, we got the kinetic results in Figures 10 and 11. Here the ethylenimine (Chemirad) was used as it came from the bottle, and we used one particular pool of WEE virus. Again, the reaction is second order as evidenced by the fact that different concentrations of virucide multiplied by time all fall on the same curve. In the absence of any statistical analysis on these data, I make no claim whether reaction rate is affected by pH. It will take many more points because the results are not in a close agreement as the results were with beta-propiolactone and ethylene oxide. To get a significant statistical result you need many more points. The reaction is about 1/10 or 1/12 as fast with ethylenimine as with beta-propiolactone. However, the total effectiveness which we talked about before is greater with ethylenimine than it is with beta-propiolactone. In a recent experiment where we used 100 milligrams per liter of beta-propiolactone at pH 6.6 and 15°C, the beta-propiolactone dropped the titer of WEE virus about twice as much as ethylenimine at the end of 24 hours. At the end of 48 hours, the ethylenimine was beginning to catch up, and at the end of about 70 hours, they were about equal, and there was still plenty of ethylenimine left. Now, I don't say an equimolar quantity of ethylenimine compared with the amount of propiolactone.

We also collected some data on inactivation of WEE virus by ethanol. Figure 12 shows the results of some of these experiments. It seems that we get at least two different rates of inactivation. In the beginning, the rate of inactivation is much more rapid than it is later on. One possible reason for a decrease in the rate of reaction is that there is a non-uniformity in the virus pool. There is a large percentage of virus susceptible to denaturation and a small percentage of virus less susceptible to denaturation. The thermodynamic characteristics of this reaction were what one would expect of a denaturation reaction. The $\Delta H^{\ddagger}$ for this reaction is around 40,000 calories, and the $\Delta S^{\ddagger}$ of activation is about 200 calories. We continued our research by investigating certain group specific reagents.




| KEY | Concentration, mg/liter | pH | Buffer, 0.15 M | Corrected Second-order Velocity Constants, liter/mole-minute |
|---|---|---|---|---|
| ○ | 15,000 | 6.60 | Phosphate | -0.339 ± 0.021 |
| □ | 30,000 | 6.60 | Phosphate | |
| ▣ | 30,000 | 7.54 | Phosphate | - - - - - |
| ■ | 30,000 | 9.60 | Borate | -0.415 ± 0.005 |
| △ | 45,000 | 6.60 | Phosphate | |

Fig. 7. Inactivation of WEE Virus by Ethylene Oxide at 15°C.




| KEY | Concentration, mg/liter | pH | Buffer, 0.15 M | Corrected Second-order Velocity Constants, liters/mole-minute | ΔH*, cal | ΔS*, cal/° |
|---|---|---|---|---|---|---|
| ● | 7,500 | 9.60 | Borate | -1.82 ± 0.08 | 16,500 | -3.0 |
| □ | 15,000 | 6.60 | Phosphate | -1.46 ± 0.08 | 16,300 | -4.2 |
| ■ | 15,000 | 9.60 | Borate | | | |
| △ | 30,000 | 6.60 | Phosphate | | | |
| ▲ | 30,000 | 9.60 | Borate | | | |

Figure 8. Inactivation of WEE Virus by Ethylene Oxide at 30°C.

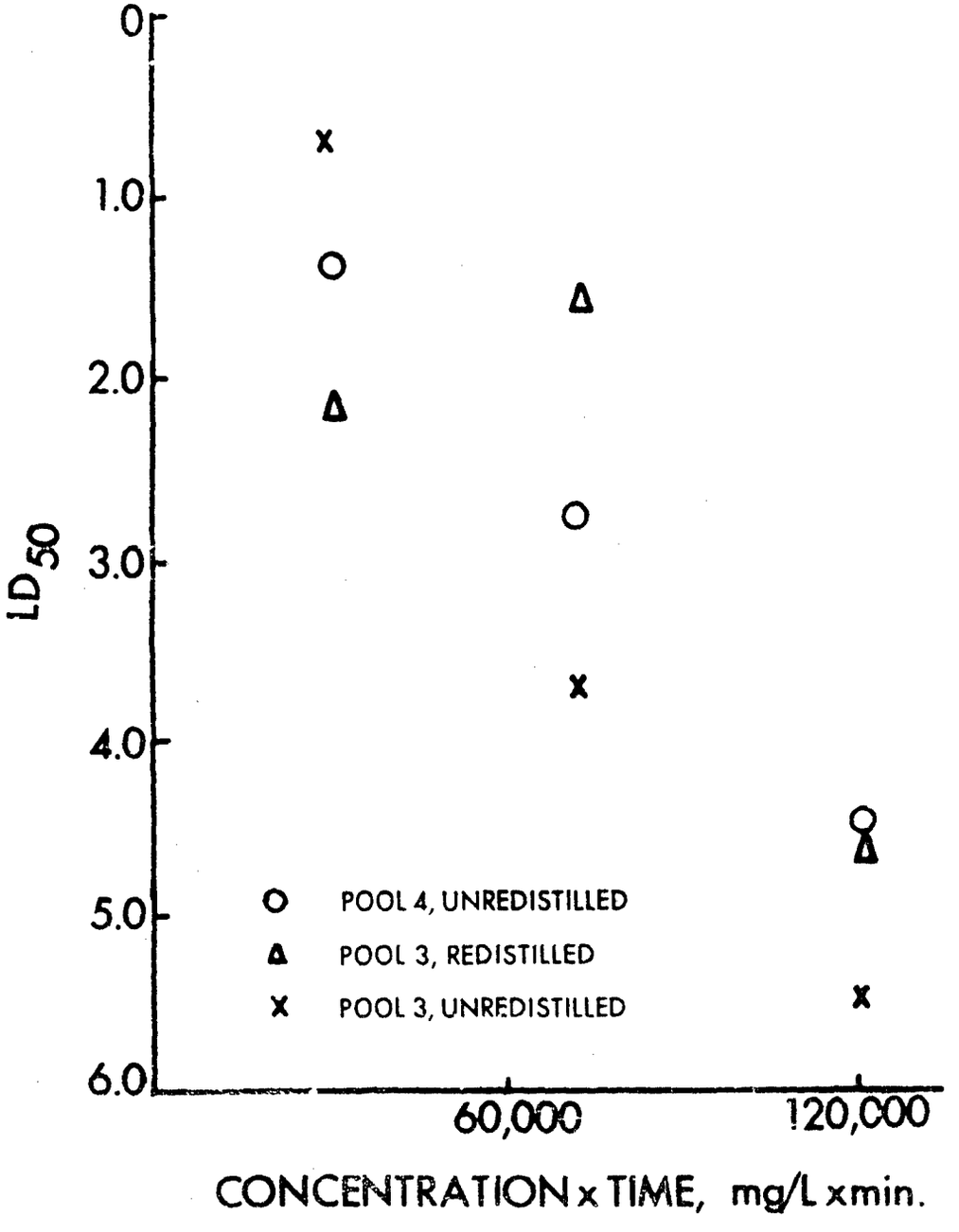
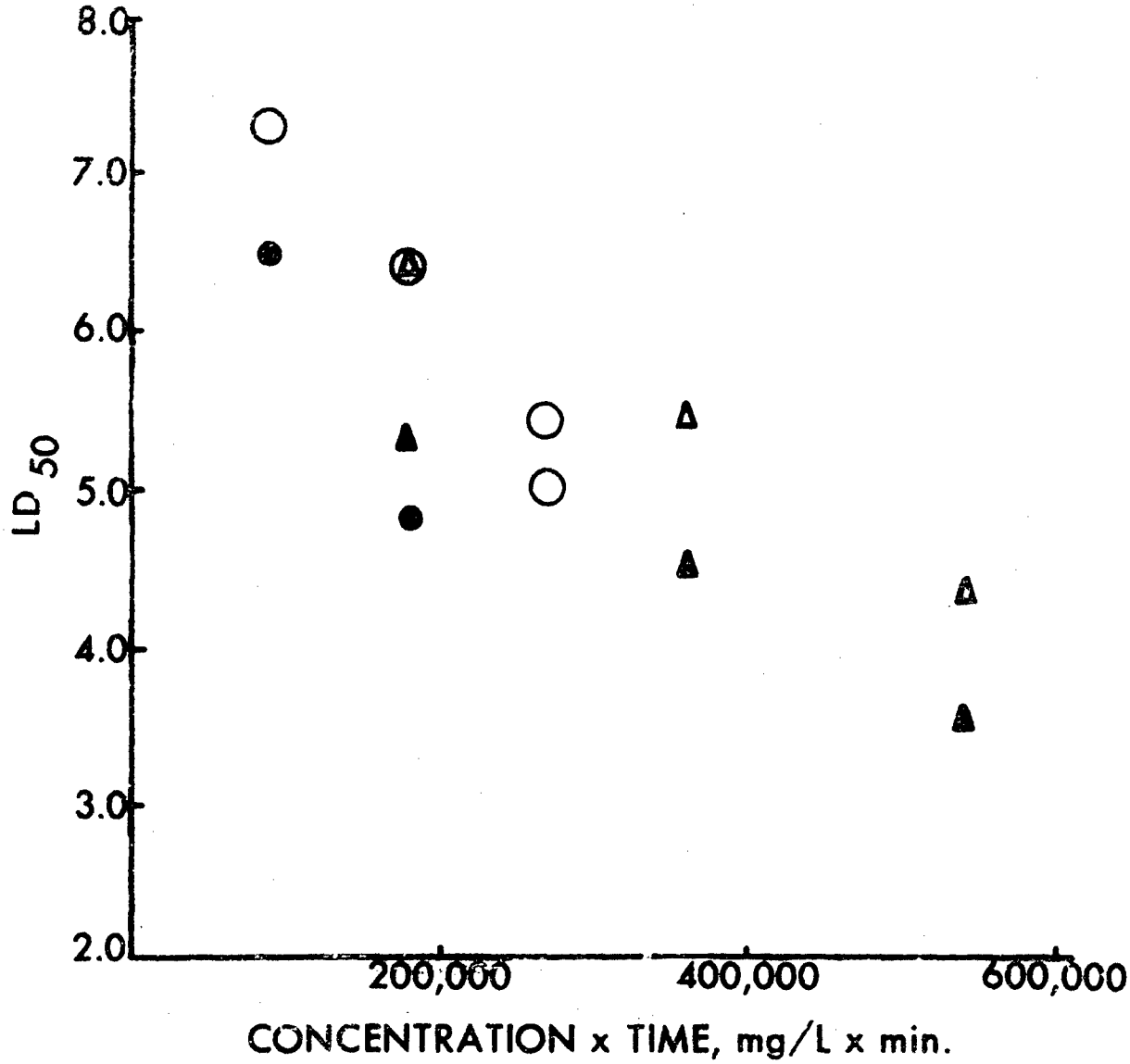
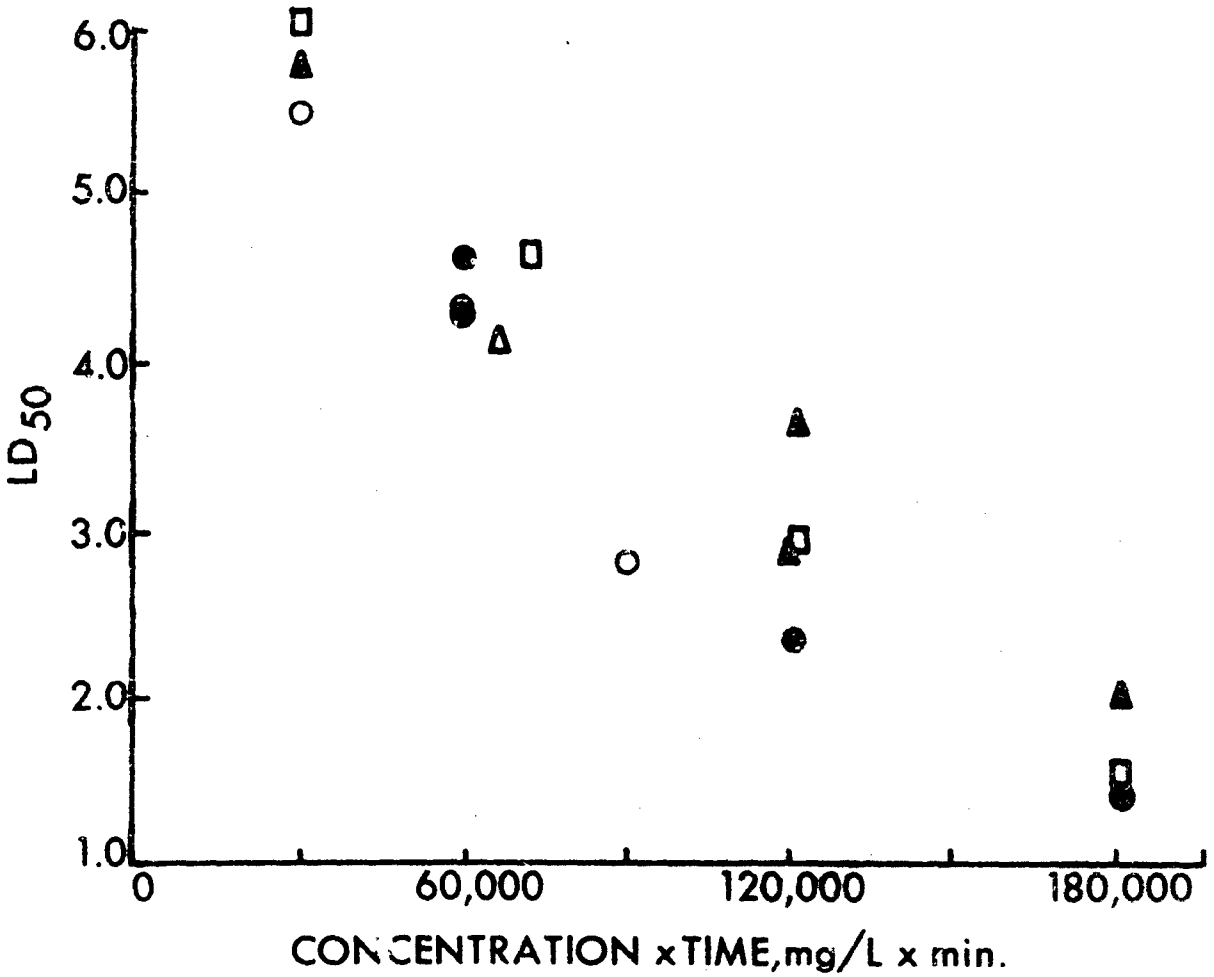



Figure 9. Inactivation of WEE Virus from Two Different Pools by Ethylenimine (Chemirad) at 30°C in 0.15 M Phosphate Buffer, pH 7.54.




| KEY | Concentration, mg/liter | pH | Buffer, 0.15 M |
|---|---|---|---|
| ○ | 6,000 | 6.60 | Phosphate |
| ● | 6,000 | 9.60 | Borate |
| △ | 12,000 | 6.60 | Phosphate |
| ▲ | 12,000 | 9.60 | Borate |

Figure 10. Inactivation of WEE Virus by Unredistilled Ethylenimine (Chemirad) at 15°C. The average titer before treatment was 7.4, and the approximate $K_2$ was 0.7 liters per mole-minute.




| KEY | Concentration, mg/liter | pH | Buffer, 0.15 M |
|---|---|---|---|
| ○ | 6,000 | 6.60 | Phosphate |
| ● | 12,000 | 6.60 | Phosphate |
| □ | 12,000 | 7.54 | Phosphate |
| △ | 6,000 | 9.60 | Borate |
| ▲ | 12,000 | 9.60 | Borate |

Figure 11. Inactivation of WEE Virus by Unredistilled Ethylenimine (Chemirad) at 30°C. The average titer before treatment was 7.4, and the approximate $K_2'$ was four liters per [illegible]ute, the approximate $\Delta H^*$, 20,000 calories, and [illegible]imate $\Delta S^*$, [illegible] per degree.

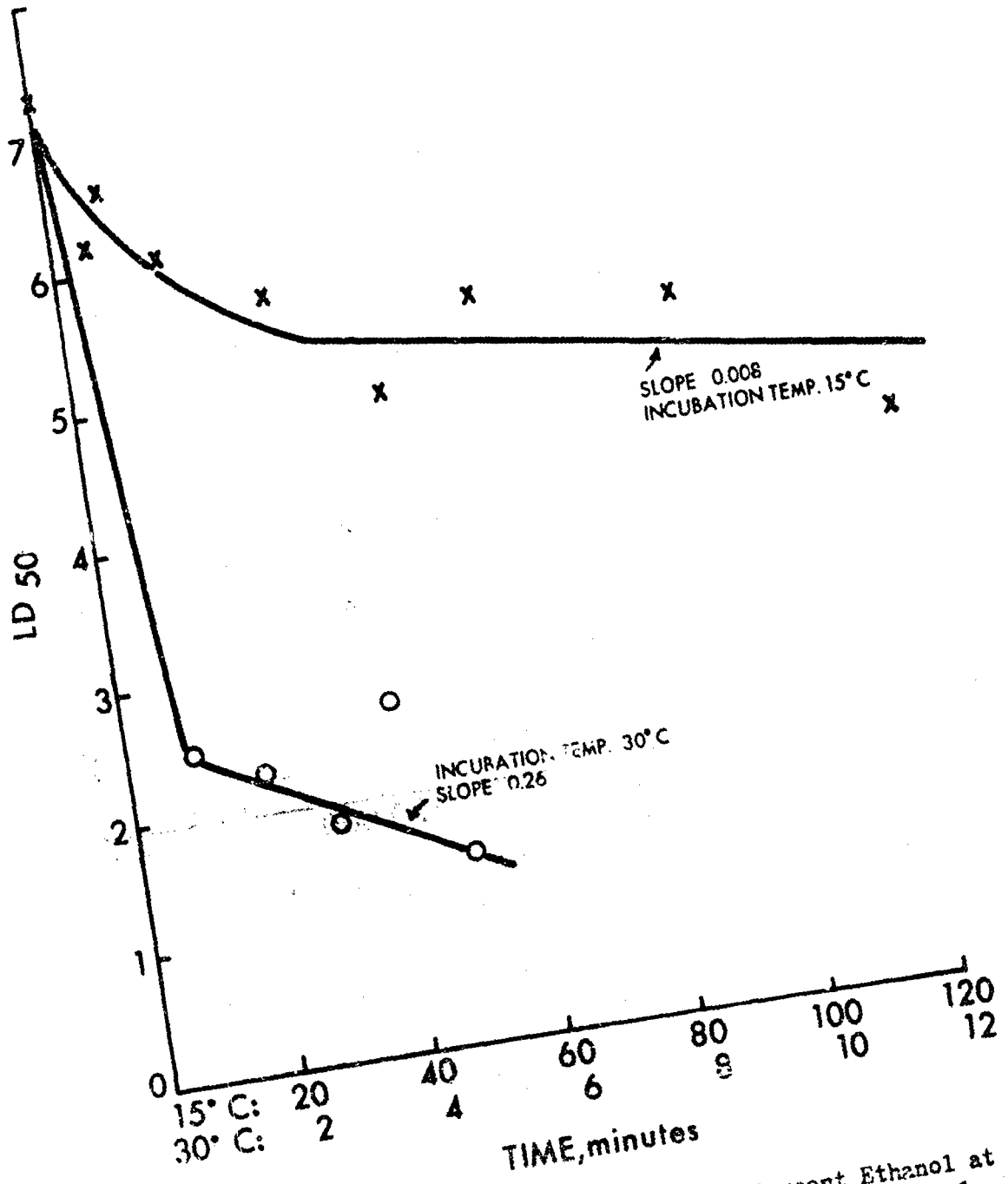



Figure 12. Inactivation of WEE Virus in 30 Percent Ethanol at pH 6.60. The solution consisted of 3 ml ethanol, 6.5 ml 0.0975 M phosphate buffer, and 0.5 ml virus suspension. The approximate $\Delta H^{\neq}$ was 40,000 calories and the ap[illegible] calories per degree.

Acetic anhydride is known to react with amino groups. We found that acetic anhydride will give inactivation of WEE virus, but it is much too rapid to measure, even at zero degrees. Dinitrofluorobenzene is not quite as specific as acetic anhydridide, but it inactivates WEE virus at a rate which we were able to measure and for which we were able to obtain the data in Figure 13. The reaction is second order as evidenced by the fact that both saturated and half-saturated dinitrofluorobenzene at any temperature fall on the same line. The thermodynamic characteristics of the reaction between WEE virus and dinitrofluorobenzene are, I think, similar to those obtained with other virucides. In general, I think it is fair to say that we cannot exclude reaction with amino groups. They possibly are responsible for the thermodynamic characteristics which we observed.

HALVORSON (Mich.): When testing a very short time interval, do you remove your material by dilution, or how do you recover your virus or your spore free of the chemical?

DUBNICK: The material is removed by dilution. The concentration of virucide is fairly low.

HEINE: Is it at all worthwhile, after you have the reaction take place with dinitrofluorobenzene, to hydrolyze the reaction mixture and use paper chromatography to find out what amino acids were affected by this dinitrofluorobenzene?

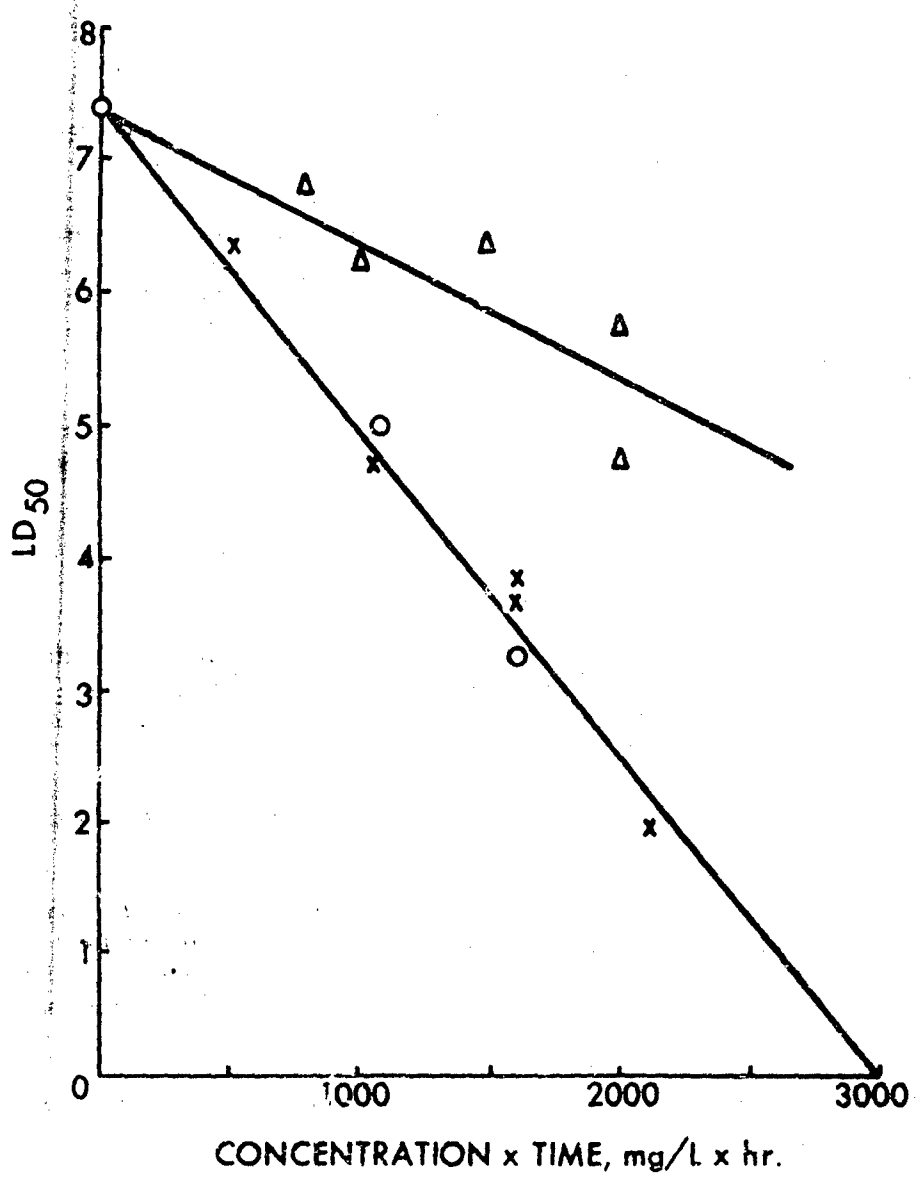
DUBNICK: You have to make sure of the amino acid content of the virus, and you would have to use a lot of virus.

SCHWARTZ: Such work can very well be done with the tobacco mosaic virus, where we know fairly well the amino acid contents, and we can produce as large an amount of viruses as you want. We could very well use a system of that nature. You can get a relatively pure tobacco mosaic virus, whereas in these systems we have much proteinaceous material.

SKIPPER: I think that those would be very interesting studies and seem to provide a very direct means for examining the problem.

HALVORSON (Mich.): Wouldn't it be better to go to the phage systems where you can get large quantities that you can purify and obtain the internal components free from the cell walls with a relatively simple technique? True, it wouldn't tell you about the animal phages, but you could get clues which you could follow from that more readily.

DUBNICK: We tried the effect of a specific reactant for sulfhydryl groups. However, when iodine is used in the presence of large amounts of potassium iodide, the agent which is supposed to be specific for sulfhydryl groups, there is inactivation, depending upon how much iodine was used and upon the temperature, but only to a point, [illegible] there is no longer any inactivation. In Figure 14, there are [illegible] short curves showing the




| KEY | Temp., °C | Concentration, mg/liter | $K_2$ | $\Delta H^*$ calories | $\Delta S^*$ cal/degree |
|---|---|---|---|---|---|
| Δ | 15 | 550 (satd.) | 2.7 | 21,000 | 33.5 |
| X | 30 | 1129 (satd.) | 15.8 | | |
| O | 30 | ½ satd. | | | |

Figure 13. Inactivation of WEE Virus by Dinitrofluorobenzene in 0.15 M Phosphate Buffer, pH 6.60.

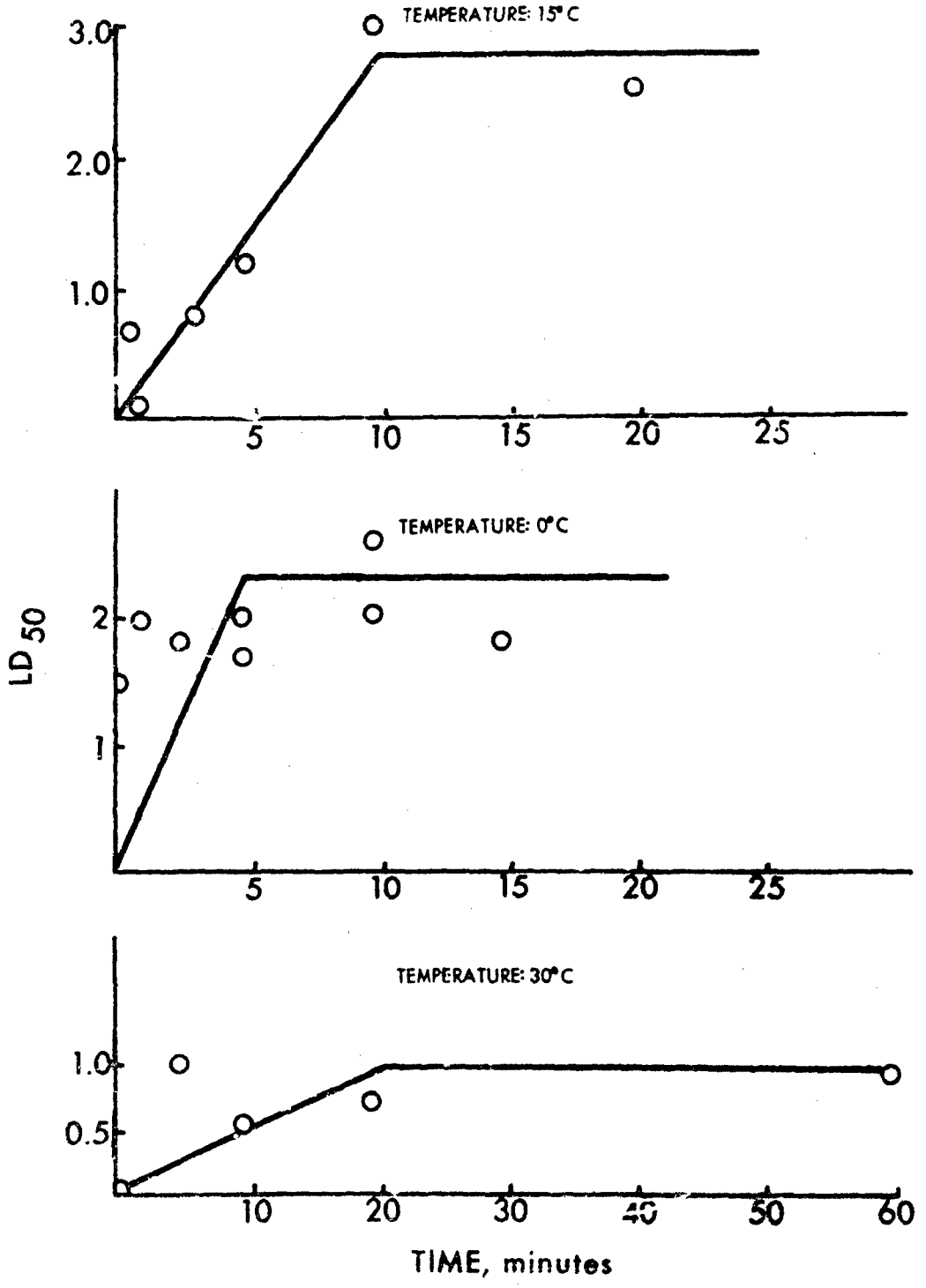



Figure 14. Inactivation of WEE Virus by 0.01 N Iodine at Various Temperatures in the Presence of 0.2 N Potassium Iodide in Phosphate Buffer, pH 6.60.

reaction between WEE virus and iodine, and illustrating the fact that there is an immediate, quite rapid decrease in titer after which there is no longer any decrease in titer. Figure 15 shows more accurate curves obtained for inactivation of B subtilis var. niger spores with iodine under sulfhydryl-inactivating conditions. It can be shown that these data can be treated as indicating an ordinary chemical equilibrium. The equilibrium constant can be calculated for the reaction, and it can be shown that the forward reaction is about 10,000 times more rapid than the reverse reaction. But with the virus, with WEE virus anyhow, it would then be impossible to reverse. It would be impossible even to attempt to reverse iodine inactivation with thioglycollate; we tried it, and there is no virus left at the end of the proper length of time. With spores, now that we have a very clean spore, it may be possible to do this without giving them a chance to germinate. Thus we might let them go for about a month, then try to reverse with thioglycollate, and see if there is actually an equilibrium reaction or if we are actually getting a reaction with the sulfhydrl group.

SKIPPER: I would like to introduce two speakers from Southern Research Institute to continue the discussion of inactivation of viruses.

SCHABEL: As you probably all know, we have a program which is primarily screening for activity of compounds against B subtilis var. niger spores that we get from Detrick, Micrococcus pyogenes var. aureus, and WEE virus. You probably have all seen the proceedings and saw the techniques that we used, and they probably do not need any review. The three sheets of Table IV list the compounds that we have found to have some activity against either B subtilis var. niger spores or WEE virus or both. I have not put on any of the data in regard to Staphylococcus vegetative cells because the vast majority, if indeed not all of these, will have some activity against that organism in vitro, and it is my opinion at this time that a good sporicidal agent is probably going to have activity against most vegetative cells. The numbers listed on the table are milligrams per liter of the active agent in the test system. The prefix "neg" means inactive; for example, under sodium peroxide, we found that it was active at 10,000 milligrams per liter or one percent, but inactive at 1,000 milligrams per liter. If there is no negative figure listed, it indicates that we haven't tested it at a concentration lower than that indicated. We have found that many of the compounds that you are all familiar with are active in vitro against B subtilis var. niger spores, and we have also found that many surface active agents have reproducible activity in the test system that we used against WEE virus. The material in Table V includes data that we have collected on combinations of (a) a compound that has activity against B subtilis var. niger spores plus (b) a compound that has activity against WEE virus. The rationale here may not be good, but we thought that we could combine chemicals which were active on the one hand against spores and on the other against viruses and get a mixture which was active against both agents and perhaps increase the activity of the compound against spores. There is not very much by way of comment that I can make on these. Essentially, we have found that none of these combinations have a striking increased effectiveness. There is one example that perhaps may be pertinent here. On page 6 of Table V, we have dichloroamine-T plus Triton X-102. Here we find that, at a level of 5,000 milligrams per liter, Triton X-102 in combination with dichloroamine-T is apparently considerably more active against WEE virus than alone.

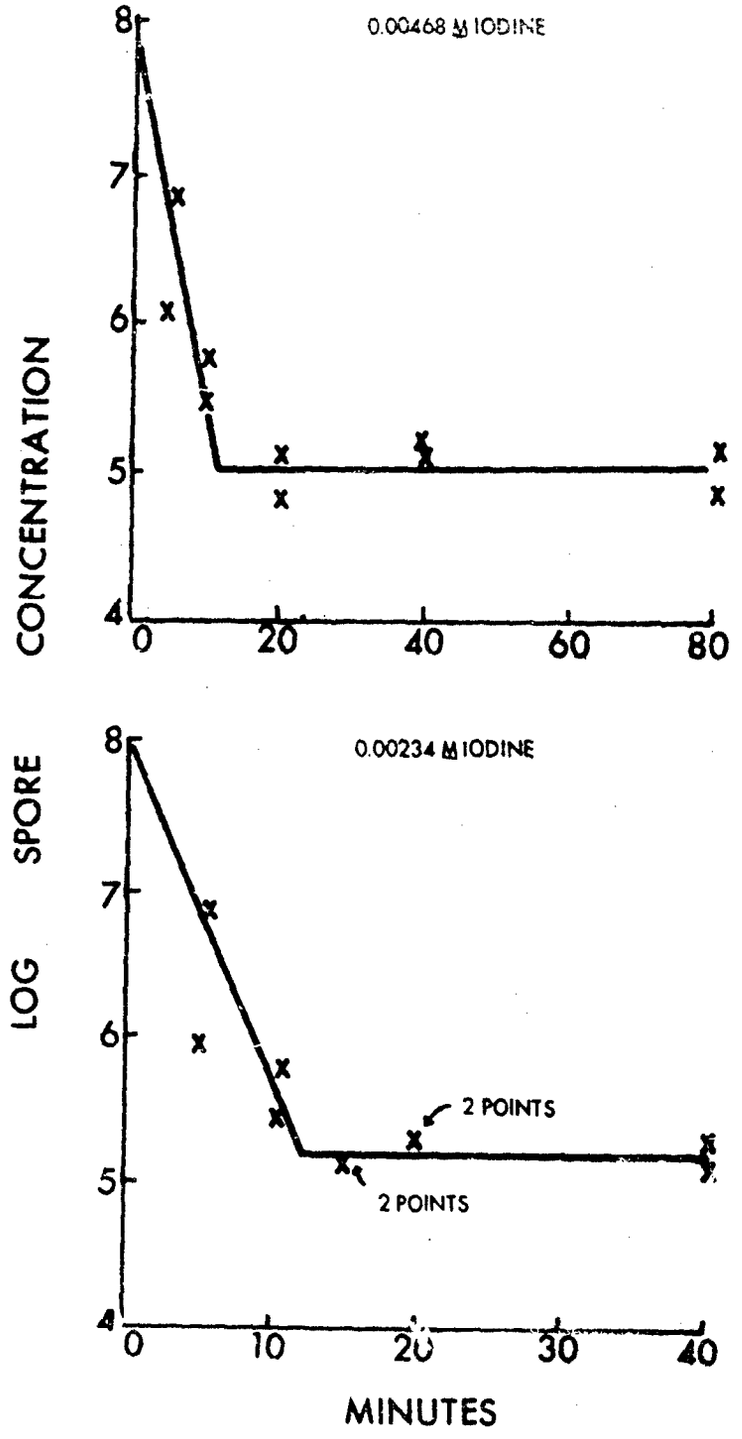



Figure 15. Inactivation of B subtilis var. niger Spores by Iodine at 15°C in the Presence of 0.012 M Potassium Iodide in Phosphate Buffer, pH 6.60.

TABLE IV. COMPOUNDS WITH ACTIVITY IN VITRO AGAINST SPORES AND/OR VIRUS

| | Active Concentration, mg/L | |
|---|---|---|
| Oxidizing Agents | B subtilis var. niger | WEE virus |
| Sodium peroxide | 10,000; neg 1,000 | 10,000 |
| Bromine | 10,000; 5,000; 1,000 | 10,000 |
| N-Bromosuccinimide | 10,000; 5,000; 1,000 | 10,000; neg 5,000 |
| N-Chlorosuccinimide | 10,000 thru 156.25 | 10,000; neg 1,000 |
| N-Bromoacetamide | 10,000; 5,000; 1,000 | 10,000; 5,000; neg 1,000 |
| Iodine monochloride | 10,000; 5,000; 1,000 | 10,000; 5,000; neg 1,000 |
| Dibromatin | 10,000; 5,000; 1,000 | 10,000; neg 5,000 |
| N-Iodosuccinimide | 10,000 thru 625 | 10,000; neg 5,000 |
| Sodium hypochlorite | 10,000 | |
| Iodine | 10,000; neg 1,000 | |
| Potassium permanganate | 10,000; 1,000 | |
| Calcium hypochlorite | 10,000; 1,000 | |
| Globaline (Tetraglycine hydroperiodide) | 10,000; 5,000; 1,000, | |
| Dichloramine T (N,N-Dichloro-p-toluenesulfonamide) | 10,000; 5,000; neg 1,000 | |
| **Aldehydes and Ketones** | | |
| Bromoacetone | | 10,000; neg 1,000 |
| Glyoxal | | 10,000; 5,000; neg 1,000 |
| Bromal | | 10,000; 5,000; neg 1,000 |
| α,β-Dibromopropional | 10,000; 1,000 | 10,000; neg 1,000 |
| Crotonaldehyde | | 10,000; neg 1,000 |
| β-Bromopyruvaldehyde | | 10,000 |
| Paraformaldehyde | 10,000; 5,000; 2,500; 1,250?act. | |
| **Acids** | | |
| Chloroacetic acid | | 10,000 |
| α-Chloropropionic acid | | 10,000 |
| Bromoacetic acid | | 10,000; neg 5,000 |
| α-Bromo-n-butyric acid | | 10,000; neg 5,000 |
| Benzenephosphonic acid | | 10,000 |
| Anthranilic acid | | 10,000 |
| Metanilic acid | | 10,000 |
| α-Bromopropionic acid | 10,000 | 10,000 |
| p-Chloromercuriphenol sulfonic acid | 10,000; 5,000; 2,500; 1,250; 625 | |

| Surface Active Agents | Active Concentration, mg/L<br>B subtilis var. niger | WEE virus |
|---|---|---|
| 2-p-tert-butyl phenoxyethyl ester of sodium sulfosuccinic acid. (Surface Active Agent BPE, American Cyanamid) | | 10,000 |
| $C_{13}H_{27}$ ester of sodium sulfosuccinic acid (Surface Active Agent TR, American Cyanamid) | | 10,000 |
| Stearyl dimethyl benzyl ammonium chloride (Triton K-60; cationic) | | 10,000 |
| Alkyl-dimethylbenzylammonium chloride (Roccal) | | 10,000; 5,000<br>1,000; neg 500 |
| NESCODYNE (West Disinfecting Co)<br>Active ingredients<br>Polyethoxy polypropoxy ethanol-iodine complex - - - - -7.75%<br>Nonyl phenyl ether of polyethylene glycol-iodine complex ----3.75%<br>provides 1.6% available iodine | | 57,500; 28,750 |
| Octylphenoxy; polyethoxy ethanol (Triton X-102; nonionic) | | 10,000; neg 1,000 |
| Alkylated aryl polyether sulfate (Triton W-30, conc; anionic) | | 10,000; 1,000; neg 100 |
| Alkylated aryl polyether sulfate, sodium salt (Triton 770, conc; anionic) | | 10,000; 1,000 |
| Alkyl aryl polyester alcohol (Triton X-138; nonionic) | | 10,000; neg 1,000 |
| Virac, n-(Caprylcolamino formyl-methyl)-pyrimidium chloride (120,000 ppm) plus iodine (30,000 ppm) (Ruson Laboratories) | | 10,000 |
| Alkylated aryl polyether alcohol (Triton X-100; nonionic) | | 10,000 |
| Di-(2-ethylhexyl) ester of sodium sulfosuccinic acid (Aerosol O T) | | 10,000; neg 5,000 |
| Sodium tetradecyl sulfate | | 10,000; 5,000; neg 2,500 |
| Cetyl dimethyl benzyl ammonium chloride | | 10,000; neg 1,000 |
| N-cetyl-N-ethyl-morpholinium ethosulfate (G-263; cationic) | | 10,000 |

| | Active Concentration, mg/L | |
|---|---|---|
| Phenols | B subtilis var. niger | MEE virus |
| 3(o-Nitrobenzylideneamino) phenol | | 10,000 |
| 4-Chlororesorcinol | | 10,000 |
| Picric acid | | 10,000 |
| Lysol | | 10,000; 1,000 |
| Phenol | | 10,000 |
| Hexylresorcinol | | 10,000; neg 1,000 |
| 2(m-Nitrobenzylideneamino) phenol | | 10,000 |
| p-Chloromercuriphenol | 10,000 thru 625 | |
| **Lactones** | | |
| β-Propiolactone | 10,000; neg 1,000 | 10,000; 5,000; 1,000 |
| **Epoxides** | | |
| 1, 2-Epoxy-3-butoxy propane | | 10,000 |
| Butadiene diepoxide | 10,000; neg 1,000 | 10,000; neg 1,000 |
| Epibromohydrin | | 10,000; neg 1,000 |
| **Heavy Metals** | | |
| Mercuric chloride | 10,000 thru 156.25 | |
| p-Chloromercuriphenol (also under phenols) | 10,000 thru 625 | |
| Silver nitrate | | 10,000 |
| **Ethylenimines** | | |
| 2, 2-Dimethylethylenimine | 10,000 | |
| N-Ethylethylenimine | 10,000 | |
| Ethylenimine | 10,000 | |
| N-Phenylethylenimine | 10,000 | |
| **Miscellaneous** | | |
| 2, 4-Dinitrofluorobenzene | | 10,000 |
| U 1023 Upjohn | | 10,000; neg 5,000 |

TABLE V. RESULTS OF THE TESTING OF COMBINATIONS OF ACTIVE COMPOUNDS FOR IN VITRO ACTIVITY AGAINST SPORES AND VIRUS

| Compound | Conc. mg/L | B subtilis var. niger | WEE virus |
|---|---|---|---|
| N-Chlorosuccinimide | 5000 | 7 | |
| | 2500 | 7 | |
| | 1250 | 7 | |
| | 1000 | 6 | - |
| | 625 | 7 | |
| | 500 | 2 | - |
| | 312.5 | 7 | |
| | 250 | 2 | - |
| | 156.25 | 5 | |
| | 125 | - | |
| Rocca1 (Alkyl-demethyl benzylammonium chloride) | 2000 | 2 | |
| | 1000 | 3 | 4 |
| | 500 | 2 | 4 |
| | 250 | 1 | >3 |
| N-Chlorosuccinimide + Rocca1 | 1000 + 1000 | 7 | 4 |
| | 500 + 500 | 7 | 4 |
| | 250 + 250 | 3 | 2 |
| N-Chlorsuccinimide | 1000 | 6 | - |
| | 625 | 7 | |
| | 500 | 2 | - |
| | 312.5 | 7 | |
| | 250 | 2 | - |
| | 156.25 | 5 | |
| | 125 | - | |
| Hexylresoncinol | 5000 | - | 6 |
| | 2500 | - | 6 |
| | 1250 | - | 6 |
| N-Chlorosuccinimide + Hexylresoncinol | 1000 + 5000 | - | 6 |
| | 500 + 2500 | - | 6 |
| | 250 + 1250 | - | 6 |

| Compound | Conc. mg/L | B subtilis var. niger | MEE virus |
|---|---|---|---|
| N-Chlorosuccinimide | 1000 | 6 | - |
| | 625 | 7 | |
| | 500 | 2 | - |
| | 312.5 | 7 | |
| | 250 | 2 | - |
| | 156.25 | 5 | |
| | 125 | - | |
| Paraformaldehyde | 10,000 | 7 | 6 |
| | 5000 | 7 | 6 |
| | 2500 | 2 | 6 |
| | 1250 | 1 | |
| N-Chlorosuccinimide + Paraformaldehyde | 1000 + 10,000 | 7 | 6 |
| | 500 + 5000 | 1 | 6 |
| | 250 + 2500 | 1 | 5 |
| | 312.5 + 1250 | 7 | |
| | 312.5 + 625 | 5 | |
| | 312.5 + 312.5 | 4 | |
| | 625 + 625 | 7 | |
| | 625 + 312.5 | 7 | |
| | 156.25 + 312.5 | 3 | |
| N-Chlorosuccinimide | 1000 | 6 | - |
| | 500 | 2 | - |
| | 250 | 2 | - |
| Glyoxal | 5000 | - | 6 |
| | 2500 | - | 6 |
| | 1250 | - | - |
| N-Chlorosuccinimide + Glyoxal | 1000 + 5000 | 4 | 6 |
| | 500 + 2500 | 3 | 4 |
| | 250 + 1250 | 1 | - |

| Compound | Conc. mg/L | B subtilis var. niger | WEE virus |
|---|---|---|---|
| N-Chlorosuccinimide | 1000 | 6 | - |
| | 500 | 2 | - |
| | 250 | 2 | - |
| Lysol | 1000 | - | 5 |
| | 500 | - | 2 |
| | 250 | - | - |
| N-Chlorosuccinimide + Lysol | 1000 + 1000 | - | 6 |
| | 500 + 500 | - | 2 |
| | 250 + 250 | - | - |
| N-Chlorosuccinimide | 1000 | 6 | - |
| | 500 | 2 | - |
| | 250 | 2 | - |
| Triton W-30 (anionic alkylated aryl polyether sulfate) | 5000 | - | 6 |
| | 2500 | - | 6 |
| | 1250 | - | - |
| N-Chlorosuccinimide + Triton W-30 | 1000 + 5000 | - | 6 |
| | 500 + 2500 | - | 6 |
| | 250 + 1250 | - | 6 |
| N-Chlorosuccinimide | 1000 | 6 | - |
| | 500 | 2 | - |
| | 250 | 2 | - |
| Triton X-102 (nonionic octylphenoxy polyethoxyethanol) | 5000 | - | 6 |
| | 2500 | - | 5 |
| | 1250 | - | 2 |
| N-Chlorosuccinimide + Triton X-102 | 1000 + 5000 | - | 5 |
| | 500 + 2500 | - | 5 |
| | 250 + 1250 | - | 2 |

| Compound | Conc. mg/L | B subtilis var. niger | WEE virus |
|---|---|---|---|
| N-Chlorosuccinimide | 625 | 7 | |
| | 312.5 | 7 | |
| | 156.25 | 5 | |
| | 78.12 | 2 | |
| p-Chloromercuriphenol sulfonic acid | 2500 | 7 | |
| | 1250 | 7 | |
| | 625 | 7 | |
| | 312.5 | 1 | |
| | 156.25 | - | |
| N-Chlorosuccinimide + p-Chloromercuriphenol sulfonic acid | 625 + 2500 | 7 | |
| | 312.5 + 1250 | 7 | |
| | 156.25 + 625 | 2 | |
| N-Iodosuccinimide | 10,000 | | 6 |
| | 5000 | | - |
| | 2500 | | - |
| | 1250 | 7 | |
| | 625 | 7 | |
| | 312.5 | 7 | |
| | 156.25 | 7 | |
| Lysol | 5000 | - | |
| | 2500 | - | |
| | 1250 | - | |
| | 1000 | | 4 |
| | 625 | - | |
| | 500 | | - |
| | 250 | | - |
| N-Iodosuccinimide + Lysol | 10,000 + 1000 | | 2 |
| | 5000 + 500 | | - |
| | 1250 + 5000 | - | |

| Compound | Conc. mg/L | B subtilis var. niger | WEE virus |
|---|---|---|---|
| N-Iodosuccinimide | 10,000 | | 5 |
| | 5000 | | - |
| | 2500 | | - |
| | 1250 | 7 | |
| | 625 | 7 | |
| | 312.5 | 7 | |
| | 156.25 | 7 | |
| Roccal (Alkyl-dimethyl benzylammonium chloride) | 1000 | - | >3 |
| | 500 | - | 4 |
| | 250 | - | 1 |
| N-Iodosuccinimide + Roccal | 10,000 | | 6 |
| | 1000 | | |
| | 5000 | | - |
| | 500 | | |
| | 1250 | 2 | |
| | 1000 | | |
| N-Iodosuccinimide | 10,000 | | 6 |
| | 5000 | | - |
| | 2500 | | - |
| | 1250 | 7 | |
| | 625 | 7 | |
| | 312.5 | 7 | |
| | 156.25 | 7 | |
| Triton W-30 (anionic alkylated aryl polyether sulfate) | 10,000 | - | |
| | 5000 | - | 6 |
| | 2500 | - | 6 |
| | 1250 | - | 5 |
| N-Iodosuccinimide + Triton W-30 | 10,000 | | 6 |
| | 5000 | | |
| | 5000 | | 6 |
| | 2500 | | |
| | 2500 | | 2 |
| | 1250 | | |

| Compound | Conc. mg/L | B subtilis var. niger | WEE virus |
|---|---|---|---|
| Dichloroamine T (N,N-Dichloro-p-toluenesulfonamide) | 10,000 | | - |
| | 5000 | 3 | - |
| | 2500 | - | - |
| | 1250 | - | |
| Triton X-102 (nonionic octylphenoxy polyethoxyethanol) | 5000 | - | >2 |
| | 2500 | - | >2 |
| | 1250 | - | 1 |
| Dichloroamine T + Triton X-102 | 10,000 + 5000 | | 6 |
| | 5000 + 5000 | 1 | |
| | 5000 + 2500 | | 4 |
| | 2500 + 2500 | 1 | |
| | 2500 + 1250 | | 2 |
| Dichloroamine T (N,N-Dichloro-p-toluenesulfonamide) | 10,000 | | - |
| | 5000 | 3 | - |
| | 2500 | - | - |
| | 1250 | - | |
| Roccal (Alkyl-dimethyl benzylammonium chloride) | 1000 | 3 | >3 |
| | 500 | 3 | 4 |
| | 250 | 2 | >3 |
| Dichloroamine T + Roccal | 10,000 + 1000 | | 6 |
| | 5000 + 1000 | 2 | |
| | 5000 + 500 | | >3 |
| | 2500 + 500 | 2 | |
| | 2500 + 250 | | 2 |

| Compound | Conc. mg/L | B subtilis var. niger | WEE virus |
|---|---|---|---|
| Dichloroamine T (N,N-Dichloro-p-toluenesulfonamide) | 10,000 | | - |
| | 5000 | 3 | - |
| | 2500 | - | - |
| | 1250 | - | |
| Triton W-30 (anionic alkylated aryl polyether sulfate) | 5000 | | 6 |
| | 2500 | | 6 |
| | 1250 | | 6 |
| Dichloroamine T + Triton W-30 | 10,000 | | 6 |
| | 5000 | | |
| | 5000 | 1 | |
| | 5000 | | |
| | 5000 | | 6 |
| | 2500 | | |
| | 2500 | | 6 |
| | 1250 | | |
| ∂-Bromopropionic acid | 10,000 | 7 | >3 |
| | 5000 | 2 | 2 |
| | 2500 | 2 | - |
| | 1250 | - | |
| | 625 | | |
| Triton X-102 (nonionic octylphenoxy polyethoxyethanol) | 10,000 | - | |
| | 5000 | - | >3 |
| | 2500 | - | >3 |
| | 1250 | - | - |
| | 625 | - | |
| ∂-Bromopropionic acid + Triton X-102 | 10,000 | 7 | 6 |
| | 5000 | | |
| | 5000 | 2 | |
| | 5000 | | |
| | 5000 | | 6 |
| | 2500 | | |
| | 2500 | 2 | |
| | 2500 | | |
| | 2500 | | >3 |
| | 1250 | | |

| Compound | Conc. mg/L | B subtilis var. niger | WEE virus |
|---|---|---|---|
| ∂-Bromopropionic acid | 10,000 | 7 | 6 |
| | 5000 | 2 | >3 |
| | 2500 | 2 | - |
| | 1250 | - | |
| | 625 | - | |
| Paraformaldehyde | 10,000 | 7 | - |
| | 5000 | 7 | - |
| | 2500 | 5 | - |
| | 1250 | 2 | |
| ∂-Bromopropionic Acid + Paraformaldehyde | 10,000 10,000 | 7 | 6 |
| | 5000 5000 | 7 | 1 |
| | 2500 2500 | 1 | - |
| p-Chloromercuriphenol | 5000 | 7 | |
| | 2500 | 7 | |
| | 1250 | 7 | |
| | 1000 | 3 | - |
| | 625 | 3 | |
| | 500 | | - |
| | 312.5 | 2 | |
| | 250 | | - |
| | 156.25 | 1 | |
| Triton W-30 (anionic alkylated aryl polyether sulfate) | 5000 | - | 6 |
| | 2500 | - | 3 |
| | 1250 | - | 3 |
| p-Chloromercuriphenol + Triton W-30 | 5000 5000 | 7 | |
| | 2500 2500 | 7 | |
| | 1250 1250 | 7 | |
| | 1000 5000 | | >4 |
| | 500 2500 | | >4 |
| | 250 1250 | | >4 |

| Compound | Conc. mg/L | B subtilis var. niger | WEE virus | |
|---|---|---|---|---|
| p-Chloromercuriphenol | 5000 | 7 | | |
| | 2500 | 7 | | |
| | 1250 | 7 | | |
| | 1000 | 3 | - | |
| | 625 | 3 | | |
| | 500 | | - | |
| | 312.5 | 2 | | |
| | 250 | | - | |
| | 156.25 | 1 | | |
| Triton X-102 (nonionic octylphenoxy polyethoxyethanol) | 5000 | - | 5 | 6 |
| | 2500 | - | >3 | 6 |
| | 1250 | - | 2 | 6 |
| | 625 | - | >2 | |
| p-Chloromercuriphenol + Triton X-102 | 1000 } 5000 } | 7 | 6 | 6 |
| | 500 } 2500 } | 3 | 6 | 6 |
| | 250 } 1250 } | 2 | 6 | 2 |
| | 125 } 625 } | 2 | 6 | |
| p-Chloromercuriphenol | 5000 | 7 | | |
| | 2500 | 7 | | |
| | 1250 | 7 | | |
| | 1000 | 3 | - | |
| | 625 | 3 | | |
| | 500 | | - | |
| | 312.5 | 2 | | |
| | 250 | | - | |
| | 156.25 | 1 | | |
| Roccal (Alkyl-dimethyl benzylammonium chloride) | 2000 | 2 | | |
| | 1000 | 2 | >4 | |
| | 500 | 2 | >3 | |
| | 250 | 1 | 1 | |
| | 125 | - | | |

| Compound | Conc. mg/L | B subtilis var. niger | WEE virus |
|---|---|---|---|
| p-Chloromercuriphenol + Roccal | 1250<br>500 | 4 | |
| | 1000<br>1000 | | >5 |
| | 625<br>500 | 3 | |
| | 500<br>500 | | >3 |
| | 312.5<br>500 | 3 | |
| | 250<br>250 | | >3 |

These numbers represent the number of logs decrease either in the viable count of B subtilis var. niger spores or in the $LD_{50}$ of WEE virus inoculated into wet chicks. A decrease of six logs indicates failure to recover virus because we use a $10^{-2}$ dilution of infected mouse brain as our stock, and it is infectious in dilutions of about $10^{-8}$. Consequently, if we decrease the infectious titer from $10^{-8}$ to $10^{-2}$, we consider that to be a six-log decrease, and that indicates that no viable virus was recovered from these mixtures. A decrease of seven logs in the count of B subtilis var. niger spores indicates the same thing because we have a suspension that titrates out in control to about $10^{7}$ spores per milliliter. I am anxious to have comments from anyone, the reaction to the possibilities or probabilities of producing or finding a usable mixture under the circumstances such as I have described. It appeared to me that perhaps the presence of a surface-active agent would increase the activity of the sporicide, perhaps by increasing contact or by some physical factor. Without belaboring these long tables, I think that there are no data present to indicate that any of these combinations are appreciably much better against either B subtilis var. niger spores or WEE virus than were the individual compounds.

There has been some activity recently by commercial firms to prepare combinations or complexes of germicidal agents. These are shown in Table VI. Wescodyne is a complex of iodine with some surface-active agent. We used this because it was sent to us from Detrick, but we also felt that it was interesting because it was along the same line of thought that we were pursuing at the time. We have tested Wescodyne against B subtilis var. niger spores and WEE virus, and in the very high concentrations which we have tested, you can see that it is quite active against both. We haven't done anything more with them than is indicated here. It is pertinent, I think, to point out that Wescodyne has a rather low pH in active solutions, particularly at the high concentrations. I think that the activity against WEE virus as indicated here can be explained. We know that WEE virus is quite susceptible to low pH's, even in the absence of any chemical inhibitor. We have also tested a commercially available combination known as Virac, which is not a complex but a mixture of a surface-active agent and iodine. We have found that this material is active, but it takes relatively high concentrations of it to inactivate either B subtilis var. niger spores or a virus. Certainly, here again, if one doesn't raise the pH of the preparation as it is available commercially when it is made up, we find that the pH is quite low, and the virus inactivating activity is considerably depressed. We have spent considerable time lately investigating a variety of halogenated compounds, particularly chlorosuccinimides, iodosuccinimides, and bromosuccinimides, and we think that they are quite interesting. Dr. Brockman has prepared some comments on the activity and possible usefulness of this type of compound, and I will let him discuss that.

ENGLEY: Wescodyne has been publicized all over the country, and I have had at least a dozen requests from state organizations to determine what it will do at 75 parts per million. Did you try it at that level?

TABLE VI. ACTIVITY OF COMMERCIALLY AVAILABLE DETERGENT-HALOGEN MIXTURES OR COMPLEXES

| | Active Agent, mg/L | B subtilis var. niger | WEE virus |
|---|---|---|---|
| **WESCODYNE (West Disinfecting Co.)** | 57,500 | 7 | 6 |
| **Active ingredients:** | 28,750 | 7 | 6 |
| **Polyethoxy polypropoxy ethanol-iodine complex 7.75%** | 14,375 | 7 | |
| | 7,187 | 7 | |
| **Nonyl phenyl ether of polyethylene glycol-iodine complex 3.75%** | 1,150 | 7 | |
| **Provides 1.6% available iodine** | | | |
| **Inert ingredients 88.5 %** | | | |

| | pH | |
|---|---|---|
| | Before Incubation | After Incubation |
| 57,500 | 2.3 | 2.5 |
| 28,750 | 3.1 | 2.9 |
| 14,375 | 6.9 | 6.7 |
| 7,187 | 7.1 | 6.9 |
| 1,150 | 7.2 | 7.0 |

| | | | |
|---|---|---|---|
| **VIRAC** | 10,000 | 1 | |
| **n-(Caprylcolaminoformylmethyl)-pyridinium chloride (120,000 ppm) plus iodine (30,000 ppm) Huson Laboratories** | 10,000[a,c] | 7 | (M pyogenes) |
| | 10,000[a,c] | - | |
| | 10,000[a,c] | - | |
| | 10,000[b,c] | 7 | |
| | 10,000[b,c] | 7 | |
| | 10,000[d,e] | | > 4 |
| | 10,000[d,f] | | 6 |

a. pH 7.2
b. pH 4.2; pH adjusted to 4 before incubation
c. Final dilutions done in sodium thiosulfate
d. Virac-virus mixture diluted in serum-saline containing 5 mg/ml of sodium thiosulfate
e. Diluted in buffer pH 7.2
f. Diluted in buffer pH 4.0

SCHABEL: No, but Bob Hoffman has. He has done a good bit, at least at lower concentrations than we have.

HOFFMAN: We have a little data of that type, but not a great deal. We found that it has little activity against B subtilis var. niger at 75 parts per million. The pH is very low, and it shows good activity against coli and staphlococcus, as you might expect.

SCHABEL: The results that were published in the promotional material were quite impressive, I thought; didn't you?

HOFFMAN: Yes.

SCHABEL: Well, we plan to do a good deal more with it, but we have just begun, and I wanted to bring it in because it is an interesting example of a surface-active agent complexed with a halogen.

HALVORSON (Mich.): Could you describe your test procedure?

SCHABEL: The test procedure? We incubate B subtilis var. niger spores as we get them from Detrick. They are not washed spores, I am sure, but they don't have anywhere near as much extraneous material as do our virus preparations. They are incubated in phosphate buffer at 37°C for ½ hours in the presence of the compound and then they are diluted out in broth. We are now using skim milk, as is the group at Detrick, for diluting.

HALVORSON (Mich.): How long does it take to make your dilutions? Is it a matter of minutes?

SCHABEL: Oh yes, it is a matter of minutes. You may have ten compounds under test, and the tenth one will have a ten-minute time lag from the time you started the dilution, but the individual dilution of a single compound or exposure system from the time of starting to the $10^{-7}$ dilution would be certainly less than two minutes.

BROCKMAN: Table VII is a summary of the types of compounds we have screened. I should like to make some observations on the in vitro activity of N-Halomides and related compounds on B subtilis var. niger and WEE virus. The halogens are known to be rather good germicides, and in aqueous media, at least, a part of their germicidal activity can be attributed to the formation of hypohalous acid, oxygen, and halic acids. The reactions described by equations 1 to 5 are known to be promoted by light.

$$X_2 + H_2O = HX + HOX \qquad (1)$$

$$2HOCl = 2HCl + O_2 \qquad (2)$$

$$3HOCl = 2HCl + HClO_3 \qquad (3)$$

$$5HOX = HXO_3 + 2H_2O + 2X_2 \qquad (4)$$

$$4HOX = O_2 + 2H_2O + 2X_2 \qquad (5)$$

(X = Br or I)

TABLE VII. SUMMARY OF CLASSES OF COMPOUNDS OBSERVED TO HAVE SPORICIDAL OR VIRUCIDAL ACTIVITY

| | Class of Compounds | Activity Against: B subtilis var. niger | Activity Against: WEE virus |
|---|---|---|---|
| I. | Organic acids | | + |
| II. | Aldehydes | + | + |
| III. | Epoxides | ± | + |
| IV. | Ethylenimines | + | - |
| V. | Lactones (β-propiolactone) | + | + |
| VI. | Oxidizing agents | + | + |
| VII. | Organic heavy metal compounds | + | - |
| VIII. | Phenols | - | + |
| IX. | Surface active agents | - | + |

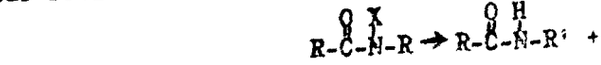
It is still open to question as to how much of the germicidal activity of halogens is due to hypohalous acid and oxygen as opposed to the halogen itself. Upon testing calcium hypochlorite, we noted, as many others have, the marked activity of the agent against microorganisms. The activity of this compound against B subtilis var. niger led to the testing of N-halogen substituted compounds for possible activity in our systems.

Chloramine-T was without activity, but dichloramine-T was moderately active against B subtilis var. niger. These compounds may be active in themselves, or, in aqueous solution, the activity may be attributed to hypochlorous acid.

Their activity as germicides has been variously attributed to the N-chloramide itself, to the hypochlorous acid formed on hydrolysis, to the halogen produced by decomposition of hypochlorous acid, and to oxygen similarly produced. We have not been overly concerned with the classification of these compounds according to their possible mode of action because we were more interested in the activity itself.

An extension of the examination of N-halogen substituted compounds showed that the N-halosuccinimides possessed appreciable activity even in rather dilute solutions. N-chlorosuccinimide and N-iodosuccinimide, for example, inhibited B subtilis var. niger spores at concentrations as low as 0.06 percent. N-bromosuccinimide completely inhibited B subtilis var. niger at concentrations of 0.1 percent, the highest dilution of this compound examined to date. The N-halosuccinimides also appear to inactivate WEE virus at the higher concentrations (one percent solutions). N-bromoacetamide and the halo-substituted hydantoins, 1,3-dibromo-5,5-dimethyl hydantoin and N-chloro-5-isopropyl hydantoin, also were quite effective in inactivating B subtilis var. niger. Examination of these structures shows that they all have in common the $R-\overset{O}{\overset{\|}{C}}-\underset{X}{N}-R'$ or $R-\overset{O}{\overset{\|}{\underset{O}{S}}}-\underset{X}{N}-R'$ grouping.

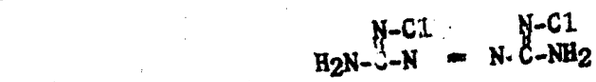
In aqueous solution, these compounds are capable of yielding the corresponding hypohalous acid and, therefore, could be classed as oxidizing agents or as halogenating agents by virtue of the reactions of hypohalous acids reviewed earlier.



(6)

We have considered these compounds of interest for several reasons: (a) their activity in the biological test systems used, (b) closely related compounds are readily available in quantity, and (c) the consideration, which may be of practical interest, that compounds of this type have previously been used to impregnate clothing.

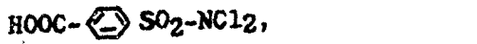
The activity of these halogen amides suggests that N,N'-dichloroazodicarboxamidine, known as azochloramide, which has been around since 1935, might be even more effective.



(7)

This compound is reported to possess the property of killing many organisms in the presence of organic matter, including serum, and is described as being non-irritating, which is interesting if true. We plan to examine this material in our test systems.

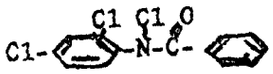
The well-known chloramide, halozone,



(8)

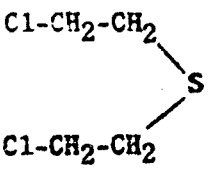
closely related to dichloramine-T, has been used for sterilization of drinking water in the field. It will be interesting to examine this compound.

The third compound, which struck me as interesting in this connection, is N-chloro-N-(2,4-dichlorophenyl)-benzamide, also known as British Impregnite, which has been used for the impregnation of clothing to provide protection against sulphur-mustard gas.

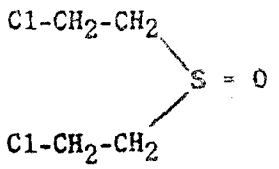


(9)

This compound inactivates sulphur-mustard gas by an oxidation to the corresponding sulfoxide and chlorosulfoxide as shown in equation 10. It is possible that this compound is capable of spore inactivation.

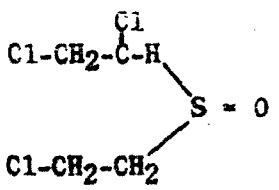


sulphur mustard



sulfoxide

(10)



Chlorosulfoxide

To summarize, the activity of halogens and inorganic hypohalites in reducing the viability of suspensions of B subtilis var. niger spores and WEE virus led to the testing of N-haloamides and N-haloimides, which were also found to be active; other similar compounds, which are readily available, have been suggested for examination.

Some of the data supporting the work I have mentioned are given in Table VIII.

SKIPPER: Did you say something about the stability of the halogenated succinimide?

BROCKMAN: I couldn't give any actual figures on that, but they are probably available. As Dr. Reddish told you this morning, calcium hypochlorite is much more effective in acid solution, and I suspect the same thing would be true with these.

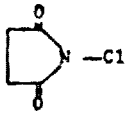
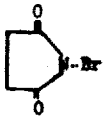
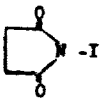
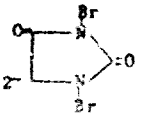
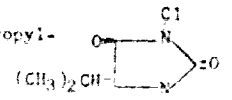
SKIPPER: Are they particularly irritating?

BROCKMAN: Yes, they are irritating.

HALVORSON (Ill.): Do these compounds prevent the spores from germinating or do they prevent the germinated spore from growing? I know that you can have either one happen. Are these compounds ones that kill the spore, so to speak, or do they permit the spore to germinate and then the vegetative cell is so sensitive that it is killed?

SKIPPER: I don't know anything about this, but could you get at that particular question by exposure and then rapid wash?

**TABLE VIII. SUMMARY OF THE ACTIVITY OF N-HALOIMIDES AND RELATED COMPOUNDS ON B SUBTILIS VAR. NIGER, WEE VIRUS, AND M PYOGENES**

| Compound | Structure | Conc., mg/l | Observed Activity[a] B subtilis var. niger | WEE virus | M pyogenes |
|---|---|---|---|---|---|
| Chloramine-T | $CH_3$-⟨⟩—$SO_2$-NHCl | 10,000 | - | - | + |
| Dichloramine-T | $CH_3$-⟨⟩—$SO_2NCl_2$ | 10,000 | +(7), +(7) | - | + |
| | | 5,000 | +(4), ±(3) | | + |
| | | 2,500 | - | | |
| | | 1,250 | - | | |
| | | 1,000 | - | | + |
| N-Chlorosuccinimide |  | 10,000 | +(7) +(7) | +(5), +(5) | + |
| | | 5,000 | +(7), +(7) | | |
| | | 2,500 | +(7), +(7) +(7), +(6) +(7) | | |
| | | 1,250 | +(7), +(7) +(7), +(7) +(6) | | |
| | | 1,000 | +(6), ±(3), - | - | + |
| | | 625 | +(6), +(6) +(6), +(6) +(6), - | | |
| | | 500 | ±(3), -, - +(4) | | |
| | | 250 | -, -, -, | | |
| N-Bromosuccinimide |  | 10,000 | +(7), +(7) | +(6), +(6) | + |
| | | 5,000 | +(7) | - | + |
| | | 1,000 | +(7) | - | + |
| N-Iodosuccinimide |  | 10,000 | +(7) | +(8) | + |
| | | 10,000 | +(7) | | |
| | | 5,000 | +(7) | | + |
| | | 1,000 | +(7) | | + |
| | | 5,000 | +(7) | | |
| | | 2,500 | +(7) | | |
| | | 1,250 | +(7), +(7), +(7), +(7) | | |
| | | 625 | +(6), ±(3), +(7), +(7) | | |
| | | 312.5 | +(5), -, +(7) | | |
| | | 156.25 | +(7) | | |
| N-Bromoacetamide | $CH_3$-C(=O)-NH-Br | 10,000 | +(7), +(7) +(7) | +(8), +(8) | + |
| | | 5,000 | +(7) | +(6), +(4) | + |
| | | 1,000 | +(7) | - | + |
| 1,3-Dibromo-5,5-dimethyl hydantoin |  | 10,000 | +(7), +(7) | +(8) | + |
| | | 5,000 | +(7) | | + |
| | | 1,000 | +(7) | | + |
| N-Chloro-5-isopropyl-hydantoin |  | 10,000 | +(7) | | |
| | | 5,000 | +(7) | | |
| | | 2,500 | +(7) | | |
| | | 1,250 | +(7) | | |

a. Inhibition of growth is represented by +. The figure in parentheses shows the logarithmic reduction of viable organisms. A reduction of 7 logs for B subtilis var. niger and of 6 logs or more for WEE virus is indicative of complete inhibition.

HALVORSON (Ill.): Yes, it can be approached. I know that some of our spores have been stored for a long time at room temperature in a dry state. We count them so we know the number of spores present, and then we also make a plate count. We find that only a small percentage of them are viable. Tested by change in permeability and change of light transmission, it was perfectly possible to notice the spores germinate, but they subsequently cannot grow. It would be interesting to know what effect these compounds do have. If it were a case of an inactivated spore that could not germinate, then we might conceivably imagine that things could happen to the spore to remove these agents so that it could germinate subsequently.

HALVORSON (Mich.): We have been very interested in the kinetics of inactivation of various strains of spores, mostly with ethylene oxide as the inactivating agent. I think we did a little bit about it last year, but it turns out that, in a number of strains, there are mixed populations, some of which are resistant and some of which are sensitive. In this last year at Detrick, they actually separated the resistant fraction from the sensitive fraction of spores by electrophoretic mobility, but we are quite interested in knowing what type of kinetics one obtains. With B subtilis var niger, you apparently have one type of population as far as ethylene oxide is concerned in separating them electrophoretically. I wonder if you have done time-course studies on some of these other compounds. Do you have any indication of a mixture of sensitivities in population, or is it just a one-time system in which you make your assay?

BROCKMAN: Well, this is actually a screening program using a fixed time exposure to the agent. It might be that we could further inactivate it at shorter times, maybe even at very short times.

MANGUN: I would like to add just one comment regarding oxidizing agents. There are many viruses that are highly susceptible to oxidizing agents, even in the presence of organic matter. It seems that WEE virus is highly susceptible, but there is a large spectrum of viruses that are virtually non-susceptible to oxidation until you go clear over to total protein denaturation. You will see this in the presence of serum, because the complete change is perfectly visible in the nature of the protein. A number of viruses we have studied can't be oxidized until you destroy everything else at the same time.

BROCKMAN: This was what seemed interesting about azochloramide, and I wondered if anyone here had had any experience with this.

REDDISH: Dr. Brockman compared to the theoretical amount of hypochlorus acid one can get from some of these agents, how is hypochlorus acid itself?

BROCKMAN: Against B subtilis var. niger, quite good. In fact we have used it as a positive control. It didn't include it in Table VIII, but I should have mentioned the fact that it is so good that we use it as a positive control.

HALVORSON (Ill.): What are the comparative concentrations required to kill spores versus vegetative cells?

**SCHABEL:** We haven't tested the vegetative cells of B subtilis var. niger.

**HALVORSON (Ill.):** What I am thinking about is the fact that anthrax spores require 250 parts per million available chlorine to kill them. I wanted to get the order of magnitude that you are dealing with.

**BROCKMAN:** Well, N-chlorosuccinimide, which requires about 0.06 percent and is 600 parts per million, kills just about as well.

**KAYE:** Dr. Phillips (Bact Rev, 16, 135-138, 1952) has prepared a little comparison table on the relative resistances of spores and vegetative cells, and in general, there is a ten-thousandfold difference in resistance to chlorine between spores and vegetative cells.

**HALVORSON (Ill.):** If you are looking for an ideal agent for spores, you have a long way to go.

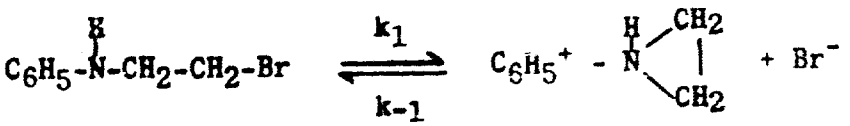
**KAYE:** Well, ethylene oxide shows only a tenfold difference between spores and vegetative cells; these alkylating agents show a very small difference. However, it takes a lot more ethylene oxide to kill even vegetative cells.

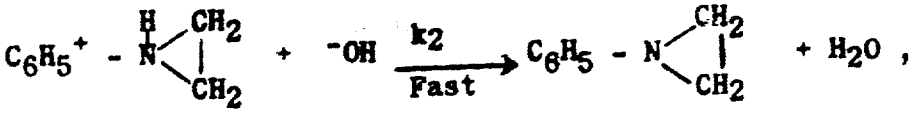
**SKIPPER:** I am sure you all realize that Dr. Brockman and Dr. Schabel have employed what we might call enlightened empiricism in their screening. We think that there are two ways to approach this problem and they have been discussed today. In one you can study modes of action, and in the second, you can use this enlightened empirical approach. I would like to be permitted to make one philosophical remark. We worked with mechanisms a lot, and we liked to think this death line is a logical approach and is really the thing to do. Then we look back in history and see that the results on the other side are much, much higher percentage-wise. It isn't a very flattering thing to say, but I can't help making the remark. Well, going from the enlightened empirical to the complete antithesis, we would like to hear from Dr. Heine, who is going to tell us about the mechanisms in the ethylenimines.

**HEINE:** We are presently studying the synthesis of various ethylenimines and their mechanisms of formation and reaction. The last time, I reported to you about the mechanisms of formation of N-phenylethylenimines from the beta-halogenated amines in the presence of hydroxide ion. This is carried out in 70 percent ethanol at 30°C. We established that the reaction product was N-phenylethylenimine and that the rate of release of bromide ion satisfies the equation

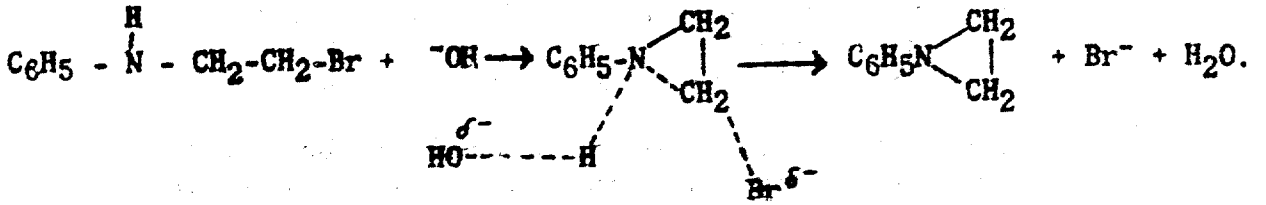
$$d(Br^-)/dt = k'(\text{bromoamine}) + k''(OH)(\text{bromoamine}),$$

where k' represents a first-order constant for an internal nucleophilic displacement of the bromine by the anilino group, that is,

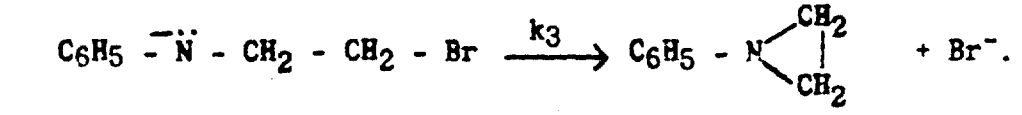




and $k_2$ represents a second-order constant for the reaction of the base with N-$\beta$-bromoethylaniline to form N-phenylethylenimine. Two mechanisms were postulated for the second-order reaction. One mechanism involved a concerted attack of hydroxide ion on the proton simultaneous with loss of the bromide ion:



The second mechanism was depicted as a two-step process involving an equilibrium between the base and the anilino group of the bromoamine to form an anilino group followed by a first-order displacement of the bromine by the anilino ion:



Since February, we have measured the rates of reaction of the various N-tolyl, para-tolyl, and ortho-tolyl beta-halogenated amines. Table IX summarizes the data on the alkaline solvolyses of N-$\beta$-bromoethyl-p-toluidine. Table X shows the dependence of the pseudo first-order constants on the sodium hydroxide concentration for N-$\beta$-bromoethyl-p-toluidine, N-$\beta$-bromoethyl-m-toluidine, and N-$\beta$-bromoethyl-o-toluidine.

As shown in the examples of Table IX, the rate of release of bromide ion follows first-order kinetics. However, as also shown in Table IX and summarized in Table X, the first-order constant increases with increasing sodium hydroxide concentration. This again is attributable to simultaneously occurring first- and second-order processes. The first order constants can be evaluated by extrapolation of the lines drawn from the [illegible] Table IX to zero sodium hydroxide concentration, while the

TABLE IX. ALKALINE SOLVOLYSIS OF N-β-BROMOETHYL-p-TOLUIDINE IN 70 PERCENT ETHANOL AT 30°C AND AT VARIOUS CONCENTRATIONS OF SODIUM HYDROXIDE

| TIME, min. | VOL. OF 0.0505 N $AgNO_3$, ml. | | $10^2k$, $min.^{-1}$ |
|---|---|---|---|
| 0.03 M N-β-Bromoethyl-p-toluidine and 0.03 M NaOH | | | |
| 10.16 | 1.08 | | 2.07 |
| 20.23 | 2.06 | | 2.11 |
| 30.28 | 2.79 | | 2.10 |
| 41.23 | 3.47 | | 2.13 |
| 62.23 | 4.32 | | 2.09 |
| ∞ | 5.93 | (mean) | 2.10 |
| 0.03 M N-β-Bromoethyl-p-toluidine and 0.12 M NaOH | | | |
| 10.16 | 1.29 | | 2.40 |
| 20.16 | 2.28 | | 2.40 |
| 30.20 | 2.06 | | 2.40 |
| 41.16 | 3.72 | | 2.40 |
| 51.20 | 4.21 | | 2.42 |
| 63.16 | 4.66 | | 2.44 |
| 75.41 | 4.93 | | 2.36 |
| ∞ | 5.93 | (mean) | 2.40 |

TABLE X. RATE CONSTANTS FOR THE FIRST-ORDER ALKALINE SOLVOLYSIS OF THE N-β-BROMOETHYLTOLUIDINES IN 70 PERCENT ETHANOL AT 30°C

| Bromoamine | Molarity, Bromoamine | Molarity, NaOH | $10^2 k_{-1}$, min.$^{-1}$ |
|---|---|---|---|
| N-β-bromoethyl-p-toluidine | 0.03 | 0.03 | 2.10 |
| | 0.03 | 0.06 | 2.26 |
| | 0.05 | 0.10 | 2.33 |
| | 0.03 | 0.12 | 2.40 |
| | 0.03 | 0.15 | 2.53 |
| | 0.03 | 0.18 | 2.64 |
| N-β-bromoethyl-m-toluidine | 0.03 | 0.03 | 1.34 |
| | 0.03 | 0.06 | 1.44 |
| | 0.03 | 0.09 | 1.53 |
| | 0.03 | 0.12 | 1.58 |
| | 0.03 | 0.18 | 1.67 |
| | 0.03 | 0.24 | 1.90 |
| N-β-bromoethyl-o-toluidine | 0.03 | 0.03 | 0.943 |
| | 0.03 | 0.09 | 0.965 |

TABLE XI. FIRST- AND SECOND-ORDER RATE CONSTANTS FOR THE RATE OF RELEASE OF BROMIDE ION FROM SOME N-β-BROMOETHYLAMINES IN 70 PERCENT ETHANOL AT 30°C

| Amine | $10^2 k$, min.$^{-1}$ | $10^2 k$, lit. mol.$^{-1}$ min.$^{-1}$ |
|---|---|---|
| $C_6H_5$-NH-$CH_2$-$CH_2$-Br | 0.94 | 2.0 |
| m-$CH_3$-$C_6H_4$-NH-$CH_2$-$CH_2$-Br | 1.30 | 2.2 |
| p-$CH_3$-$C_6H_4$-NH-$CH_2$-$CH_2$-Br | 2.00 | 3.6 |
| o-$CH_3$-$C_6H_4$-NH-$CH_2$-$CH_2$-Br | 0.94 (approx.) | 0.36 |

second-order constants can be estimated by the slopes of the lines. These values are presented in Table XI together with the previously determined constants for N-$\beta$-bromoethylaniline.

With the unsubstituted bromoamine, we get a rate constant of 0.94 x $10^{-2}$ $min^{-1}$ for the first-order constant and 2.0 x $10^{-2}$ lit $mol^{-1}$ $min^{-1}$ for the second-order constant. When we put a methyl group in the meta position, it donates electrons to the ring and, therefore, should make it easier for the nitrogen to displace bromide. Consequently, the first-order rate should go up; it does, for we get a rate constant of 1.30 x $10^{-2}$ per minute. When we put the methyl group in the para position, it will pump electrons even more readily than in the meta position, and we would expect that this first-order reaction would go up still more. We get a constant of 2.0 times $10^{-2}$ reciprocal minutes. Finally, if we put a methyl group in the ortho position, we would expect that the methyl group would get in the way of the nitrogen as it tried to cyclize thus sterically hindering the reaction. We find this to be the case; we get about 0.34 x $10^{-2}$, exactly the same as the unsubstituted ones. Thus, in this case, the effect of the methyl group pumping in the electrons and making the nitrogen more basic is counteracted by the methyl group getting in the way of the nitrogen as it tries to cyclize.

A very interesting observation concerns the constants we got in the second-order reaction, 2.0 for the unsubstituted, and 2.2 if we put the methyl group in the meta position. The second-order reaction rate goes up. In the para position, we get 3.6, and in the ortho, 0.36. Now as far as these compounds are concerned, it eliminates the first mechanism we proposed for an aniline ion forming and subsequently displacing the bromide; it means that a concerted attack is taking place where the hydroxide ion attacks the proton simultaneously with the nitrogen displacing the bromine.

LEONARDS: It might be worthwhile pointing out to the more biologically oriented people in the group that these reaction mechanisms may not help us get at the reason why these compounds might be effective against spores. But they are of value in helping synthesize new compounds in this group in better yields in the hope of finding compounds better than ethylenimine, itself, as a sporicidal agent. That is where the practical value of this work lies. The real purpose is to obtain new compounds that might have activity and properties better than ethylenimine, and these studies are really designed to help obtain these new compounds.

SCHWARTZ: Is anything known about the influence of substitution on the toxicity of these compounds?

HANGUN: There is some information about these compounds. In general, the toxicity rises with the reactivity until you get such rapid reactions that the compounds just don't get anywhere in the body, and then it drops again.

## III. MINUTES OF THE SECOND SESSION

**KAYE:** I would like first to welcome Dr. H. O. Halvorson of the University of Illinois and turn the proceedings of this session on spores into his capable hands.

**HALVORSON (Ill.):** Well, I take it you would like a progress report on our work at the University of Illinois. Our work has dealt with Bacillus terminalis, the name of which has been changed to B cereus var. terminalis. We have also done some work on the anaerobes and botulinus, types A and B. An interesting observation was that, previously, we have been able to get our spores to germinate with L-alanine and adenosine at room temperature in distilled water. However, we received a large batch from Detrick, and these spores would not germinate under these circumstances. Even after heatshocking, they would not germinate unless we added phosphate, borate, or carbonate. We suspected heavy metal contamination and so dialyzed them against versene. After dialyzing, they then germinated normally. We had done a few preliminary studies on terminalis and found that Mg, Co, Fe, Cr, and Ni ions all interfered with germination. We don't know the limit of the concentrations yet, but we get inhibition at very low concentrations.

We have made a few preliminary studies on the work that Brooks Church and Harlyn have done at Michigan on enzymes that can be activated by heat-shocking. At Michigan, they find that they can take spores and heatshock them at 65°C for an hour or more, and that they then are enzymatically active in the oxidation of glucose. In our experience with these spores, we got no activity unless we added yeast extract to the media. Normally, if you add yeast extract the spores will germinate. We found that we can prevent this germination by adding a fairly high concentration of phosphate. We don't know what ingredient in yeast extract is responsible for this germination. It is not any of the common cofactors. We have tested ATP, coenzyme 1, coenzyme 2, and pyridoxine with phosphate, and so far none of them can replace yeast extract. We don't know what the factor is; it may perhaps be a metal, but we will have the answer, I think, shortly.

One of the problems that we have been concerned with is the effect of moisture activity on the germination of spores. Some of this work has been published. The technique we are now using is to prepare spores standing in adenosine and L-alanine in the cold room (where they can't germinate), place them on glass slides, dry them thoroughly, and then take these slides and put them in desiccators over sulfuric acid of various concentrations to give different relative humidities. We then remove the slides and screen them to see if the spores have germinated in order to find the limit of moisture concentration which will allow germination.

Another technique we have used is to clean the spores in a solution containing alanine and adenosine with various concentrations of sucrose which will alter the moisture activity. We find that they are working with a mixed population. A few of the spores, from 10 to 15 percent, will germinate with a relatively small moisture activity equivalent to a vapor pressure of about 96 percent of saturation. The other 85 percent of the spores will not germinate until the moisture activity is equivalent to 99 percent or better. But we have a mixed population, and we are anxious to find out whether this is due to difference in fat content of these spores, which is something we have not done before.

One other observation which is of interest (and essential for inactivation of spores) is that we tested the effect of rancid fatty acid on the germination of spores. We found that you could test the production of a colony and observed fatty acids interfering with germination. In the presence of the rancid fat, you get fewer colonies produced than you do without the rancid fat. What is actually happening is that the rancid fatty acid is destroying or killing the newly formed vegetative cells.

Here we have two different types of mechanisms for spore inactivation. In the presence of heavy metals, spores cannot germinate and, therefore, cannot produce a colony. On the other hand, as with these fatty acids, you may have a toxic ingredient in the presence of which the spore can germinate but can't grow. In studying the compounds that inactivate spores, it may be important to distinguish between these effects.

We also found that the viability of spores which have been standing open to the air and in a dry state goes down to around 30 percent after three years; yet these spores all germinate with adenosine and alanine. You have the spores germinating in buffer, but the resulting vegetative cells cannot grow. Here, we have spores inactivated by something that was produced by this long period of standing.

For the anaerobes, we have been able to develop a clear liquid media which gives us 100 percent sporulation in 48 hours with the anaerobes Clostridium roseum, Botulinum type A, and Botulinum type B. For a long time, we had a great deal of difficulty in harvesting these spores produced because it appeared to us that the spores were germinating in the same soup in which they were formed. This apparently was caused by (a) growing the spores without taking any special precautions for anaerobic or aerobic conditions, and (b) a layer on the surface of the medium in which the normal ingredients were used up. When they were used up in the rest of the medium it did not interfere with germination. We grew these spores under anaerobic conditions, producing anaerobiosis by simply bubbling gas from the gas jet through the medium, setting it on a magnetic stirrer, and keeping it stirred. We found that we got a much higher yield of vegetative cells and 100 percent sporulation in 48 hours. Apparently we have used up all of the nutrient so that the cells can no longer germinate. By this method we have been able to get these clean spores, practically pure, from vegetative cells. In all three of

these anaerobes, we have been able to get them to germinate within two to five minutes with a mixture of L-alanine, L-phenylalanine, and arginine. You can follow the germination by change in light transmission as with aerobes. Germination will not take place unless you have complete anaerobic conditions; you get that by adding sodium thioglycollate to this medium. We know very little further about the mechanisms of this germination except that we do know that these anerobes do not contain a racemase and, therefore, the L-alanine does not convert to D-alanine. We also observed that, during the course of germination, these spores use up a large amount of the alanine which is in the medium, which is different from that observed with the aerobes. When the aerobes germinate, you find practically all of the alanine left in the medium, and some of it has been converted to D-alanine. Here it is not. A large amount of it has been used up.

If you hold these spores at 65°C, they fail to germinate, and the mechanism that uses up alanine does not operate at 65°C. When you reduce the temperature again to 30°C, the spores germinate rapidly, so the mechanism that is involved is not destroyed at 65°C.

SKIPPER: In germinating the spores in which there is a requirement for alanine and adenosine, ATP will not substitute for adenosine, will it?

HALVORSON (Ill.): No, it will not, but my statement was that if you heat these spores at 65°C for an hour, you activate enzymes that will oxidize glucose. This enzyme apparently requires a factor from yeast extract, and this factor is not ATP, ADP, or pyridoxine.

SKIPPER: In a germination, will other adenines substitute for adenosine?

HALVORSON (Ill.): No.

SKIPPER: This is really quite fascinating. I have never heard of anything that adenosine would do that, in some degree, one of these others would not.

HALVORSON (Ill.): One of the former students who was at this meeting last time, Dr. Lawrence, who is not here now, has apparently encountered an enzyme in these spores that decomposes adenosine to adenine and phosphoribase.

SKIPPER: Apparently it does not go the other way.

HALVORSON (Ill.): These two will not substitute for adenosine.

SKIPPER: That is very fascinating. I assume you cannot substitute for alanine the alpha hydroxy or alpha keto acid?

HALVORSON (Ill.): We have not found any substance to replace alanine. When you heat shock the spores for a long time, however, they will then germinate with adenosine alone. But I suspect strongly that there is a small amount of alanine being formed within the spore during the heating.

HALVORSON (Mich.): The thing that is complicated in this regard is that the amount of alanine and the amount of adenosine have reciprocal relationship to each other: If you use a high amount of adenosine, you can get away with very little alanine; if you use very little adenosine, you need a large amount of alanine. It is difficult to say what is involved. We have done some tracer work with alanine, and we find that a very small amount is bound. It requires aerobic conditions, but we can't detect any metabolism of these compounds.

HALVORSON (Ill.): One other fact is that these spores would not germinate even with alanine and adenosine at 65°C. If you cool them down to 30°C, they will germinate rapidly providing the temperature has not been kept at 65°C too long. The enzyme that decomposes adenosine is still active at 65°C, and if you keep it at that too long, your adenosine is all gone, and you have to add more adenosine before you can make them germinate.

STIM: On the basis of your studies on the germination of Detrick spores when you found that metals interfere with germination, would you recommend dialyzing all your batches of spores before running sporicidal tests?

HALVORSON (Ill.): It may well be that, if we had taken the same precaution in washing these spores as we did with the first batches, we might not have observed this phenomena. The preparation looked so clean to us that we dispensed with the special cleaning. The medium is removed just by simple washing. I might tell you also a method used recently for cleaning spores which worked out very well in separating the spores and the vegetative cells debris. We make a series of layers of sucrose solutions, 40 percent, 20 percent, and 10 percent in a centrifuge cup. First you put in the 10 percent solution, and then inject underneath that the 20 percent solution, and underneath that the 40 percent solution, and let that stand in the cup overnight. Then layer your spores on top of that and centrifuge. The spores will go clear to the bottom, and the debris will remain in the 10 percent solution. The spores you get at the bottom are very clean.

HALVORSON (Mich.): We ran across a rather nice trick. If you put B subtilis var. niger in a flask in an icebox or cold room for several weeks and then siphon off the top layer, you will find the layer just below is very clean. By repeating this for two or three periods, you can obtain very clean spores of the niger variety. It doesn't work as well with others, but with that particular strain, it works very well.

STIM: Is there any difference in resistance between different layers of spores?

HALVORSON (Mich.): No, that has been a point of some concern to us, that maybe we are throwing away one fraction and purifying another, which may not be the whole story. We haven't really looked into that, although in the past we have washed up the entire material, and we know our present resistance [illegible] with that obtained before.

MAYE: Dr. Leonards observed something similar by accident in the preparation of some spores.

LEONARDS: Yes, and I think this is the same phenomena. All we did was let the suspension stand for awhile and that autolyzed the vegetative cells, but I don't think there were any metals involved. The difficulty in letting the spores stand for a long time if you are trying to make 10 pounds of spores is the fact that you are holding up a large tank for six or eight weeks. I guess it is all right on a small scale.

SKIPPER: How general is this adenosine and L-alanine requirement for sporulation? I mean is it required for all the aerobes to germinate?

HALVORSON (Ill.): According to the English literature, they claim to have obtained germination with glucose without the adenosine, but we have had to use adenosine and alanine.

SKIPPER: I just wondered if this has a practical application. I don't know much about this, but I wonder (if you want to prevent germination) whether some of the materials that are antagonists might not be active enough in small concentrations to stop the germination.

MANGUN: This matter of whether your spores are killed before they can germinate or after they can germinate and can't reproduce is very interesting. How far does the data go in studying different types of compounds? I had in mind, specifically, whether these agents that have been studied intensively, such as the mustards, prevent germination or not? If they don't prevent germination, then one can assume they are acting in the same way as they are acting in vegetative cells.

CHURCH: In our studies with ethylene oxide, we inhibited germination of the agent. We attempted to germinate spores after varying intervals of exposure to ethylene oxide. The curve we got, kinetics of the inhibition to germination, was identical with the curve we got when the treated spores were placed on the agar plate. Or in other words, if spore growth would take place, so would vegetative cells. This indicated then that in all instances it was the germination phase that was being hit. In other words, they were not going to the germinated spore and then being inactivated. Whatever site is being hit with the ethylene oxide present happened in the ungerminated spore. It may be that the same site is affected in the vegetative cell, too.

MANGUN: I don't know what germination is, except that it involves some type of de-polymerization or uncoiling, but as far as we know at the present time, it is not involved in the nucleic acid system.

HALVORSON (Mich.): The initial adsorption may require a higher concentration in the spore than in the vegetative cell because, I think, the surface comes into play. We have a fair amount of fat on the surface of spores, and so it may require a higher concentration to bind it and hold it. I don't think the concentration difference would tell you very much.

We have been involved for about three years now in working with Detrick spores. We started out on the effects of ethylene oxide and diverted to the mechanisms, but I'll leave the mechanisms until this afternoon. We have been following the behavior of ethylene oxide on spores, trying to get some idea of the action of the compound. Our intent was to use labelled ethylene oxide to try to find out where the chemical is adsorbed, but we ran across a phenomenon which has sidetracked us now for two years which I would like to discuss in a moment. In studying the inactivation of B terminalis spores by ethylene oxide at 5°C, we obtain the same type of curve whether we count the number of spores which have germinated after various exposures to ethylene oxide, do viability on a plate, or measure the activity of racemase enzyme. We have a very rapid period followed by a slower period; this has been done many times and, as near as we can tell, appears to be two exponential functions. We checked the inactivation of many species of spores. We seem to get this type of break only with two strains: B polymyxa and B terminalis. For some reason, we became suspicious that the fat content of the spore might be involved. We tried to test whether or not fats were involved in this and whether this may represent a mixed population. We extracted spores with methanol and chloroform and then tested the population. B terminalis gave us, on fat extraction, a very rapid kill equivalent to the initial slope of the unextracted population. Following this line of thought, if we did have a mixed population in which fat was involved in some way or another, this might be a surface property of the spore, and perhaps we might pick up this difference in difference of surface charge. We used a technique whereby one can measure the individual mobilities of spores in the electrophoretic apparatus with a microscope. We carried out mobility measurements on large numbers of spores, and found that, from the original population, we could construct a histogram in which we have two populations; a small population and a large one. If we fat-extract these spores and then run an electrophoretic histogram, we lose our fast travelling population which corresponds to the resistant fraction. We thought perhaps that, if fat were involved and we were able some way or other to increase the fat content, this might be reflected in a more resistant population. We found that by incorporating glycerol into the medium we could obtain what we called "fat" spores. To give you rough idea, B terminalis is 5.4 cubic microns in size, normally. Grown in the presence of glycerol, this increases to about 15 cubic microns. The fat content, that is, the percent of the material which can be extracted, rises from 4.5 percent to 30.8 percent. In B polymyxa, which also shows an inhomogeneous population in resistance and mobility, our size goes from about 5.1 to about 25 cubic microns, and the fat content rises from 8 percent to 63 percent of the spore. The strains of B cereus, B subtilis var. niger, and another strain of B subtilis, all of which appear to have homogeneous ethylene oxide kinetics, all appeared homogeneous in mobility, and did not become larger when grown in glycerol. We analyzed the fat which one can obtain from these spores and analyzed for fatty acids and glycerol as components of the fat. Actually, in the literature, there is really very little data on Bacillus fat analysis. We found that the major component of the fatty acid was acetic acid with a little bit of butyric and a very little bit of [illegible]. We have collected a fair amount of the non-saponifiable fraction, but at the moment, we have no knowledge of what it may be. It is quite obvious that it will be a triglyceride because the triglycerides are water-soluble.

We have tried to see if the fat can be involved in the ethylene oxide inactivation by doing reconstruction experiments. That is, we determined the normal inactivation curve obtained in distilled water, added fat to the suspension of spores, and ran an ethylene oxide inactivation.

In other work, we found that B anthracis contains an alanine racemase, and the alanine racemase is more active than in any of the other six strains of Bacillus. Whether this is related to pathogenicity or not, I don't know. One can purify anthrax in a manner similar to the other strains whose problems are really very, very similar, and I think a good deal of information can be transferred.

REDDISH: Were these heated or unheated spores? What was the medium used for growing the spores, and at what time and temperature did you grow them before you harvested?

HALVORSON (Mich.): Well, the cleaning of spores is almost a different problem for each strain. It has to be worked out individually, but the general overall picture with the aerobic spores is one of finding an inorganic medium that will stimulate sporulation. These are vigorously aerated at 30°C. To get an optimal spore crop, it is really necessary to carefully watch the percentage of spores being formed in a growing culture. There is about a one-to-two-hour period during which one should harvest to give maximal yield. Following that period you get lower yield. Spores are different when grown under different conditions. One problem is to get a good yield and the second is to get uniform crops.

I think the only thing we have in this vein comes from some enzymology studies we did some years ago when Dr. Harrell was with us. We were interested in this question: Does the ethylene oxide get in and inactivate the enzyme inside the spore or is it a surface effect only? We grew spores in the presence of penicillin, sporulated them in vitro, and tested the intact clean washed spores. These had no penicillinase activity. However, when we ruptured these cleaned spores and tested the extract they were penicillinaise positive. The strains grown in the absence of penicillin do not produce penicillinase so that these spores metabolically may differ, however, we just haven't systemically worked with that.

SKIPPER: One other question - you say that the racemase activity parallels the killing?

HALVORSON (Mich.): Yes

SKIPPER: How about other enzymes? Are there any other enzymes that follow along that line?

HALVORSON (Mich.): We thought the enzyme would be a good one to follow as a criterion of inactivation, and then we spent quite a bit of time trying to decide whether the racemase was at all involved in germination and we came to the conclusion it was not. So we gave up racemase. Then we tried to look for other enzymes, but unfortunately we are coming to the end of the contract and are closing the door. But I think it would be worthwhile to test some of these other enzyme systems on inactivating agents.

CHURCH: I wanted to discuss the inactivation of the enzyme, the kinetics of which are similar to kinetics of inactivation of germination and viability. We thought that perhaps you could determine these racemase inactivation rates by ethylene oxide and get some correlation with inactivation. However, when racemase was ruled out as an enzyme responsible for germination, we decided that possibly there were other things in the spores to be looked at. The literature is very discouraging on this point.

We tried to prolong the period of heatshocking to see if this had any effect on glucose oxidation. We were also aware of the fact that terminalis spores did not germinate in the presence of glucose. In our system, there is no germination, and the intact spores were exposed to 65°C for one hour. Figure 6 demonstrates the oxygen uptake on this type of spore at various times of heatshocking at 65°C. There is no endogenous respiration. These spores are placed in a vessel with phosphate and glucose, and that is all. Following the heatshocking and within 15 minutes' time, the oxygen uptake is very slight. However, if the time is increased to 60 minutes, the maximum oxygen uptake is a very remarkable amount. At the same time the endogenous respiration increases substantially. We heatshocked the spores for an hour, stored them in the icebox, and then tried to see if there was any effect of time after heatshocking in the oxygen uptake in the presence of glucose. Now, if the spores are heatshocked and immediately placed in the washing flask for a period of 0 to 2 hours afterwards, we still obtain maximal oxidation. However, if these spores stand for 48 hours in the icebox and then are tested in the washing flask, there is essentially no oxygen uptake, and what we originally got in 15 minutes is gone besides. However, if there is an additional heatshock added to this 48-hour suspension, additionally heatshocked for another hour at 55°C, it is practically returned, but we are never able to return it completely to the optimum condition. Within 24 hours, we are nearly able to return the oxidation activity to the maximum but not completely. And if we wait one week and re-heatshock, it is about gone. In the way of explanation, I have noticed that possibly after we heatshock for an hour, we did stimulate significantly the endogenous respiration so that the spores may be utilizing intermediates that are not then being replaced by adding glucose after a week. We can show that heatshocking alone is not necessary to demonstrate oxidation in the spores because, if we take the ungerminated spore and heatshock it, we have a curve like that shown in Figure 17. Both the germinated spore and the ungerminated spore demonstrate very closely equivalent oxygen uptake. Figure 18 shows a series of substrates, and other carbohydrate intermediates we used [illegible] other substrates and pathways would be possibly involved.

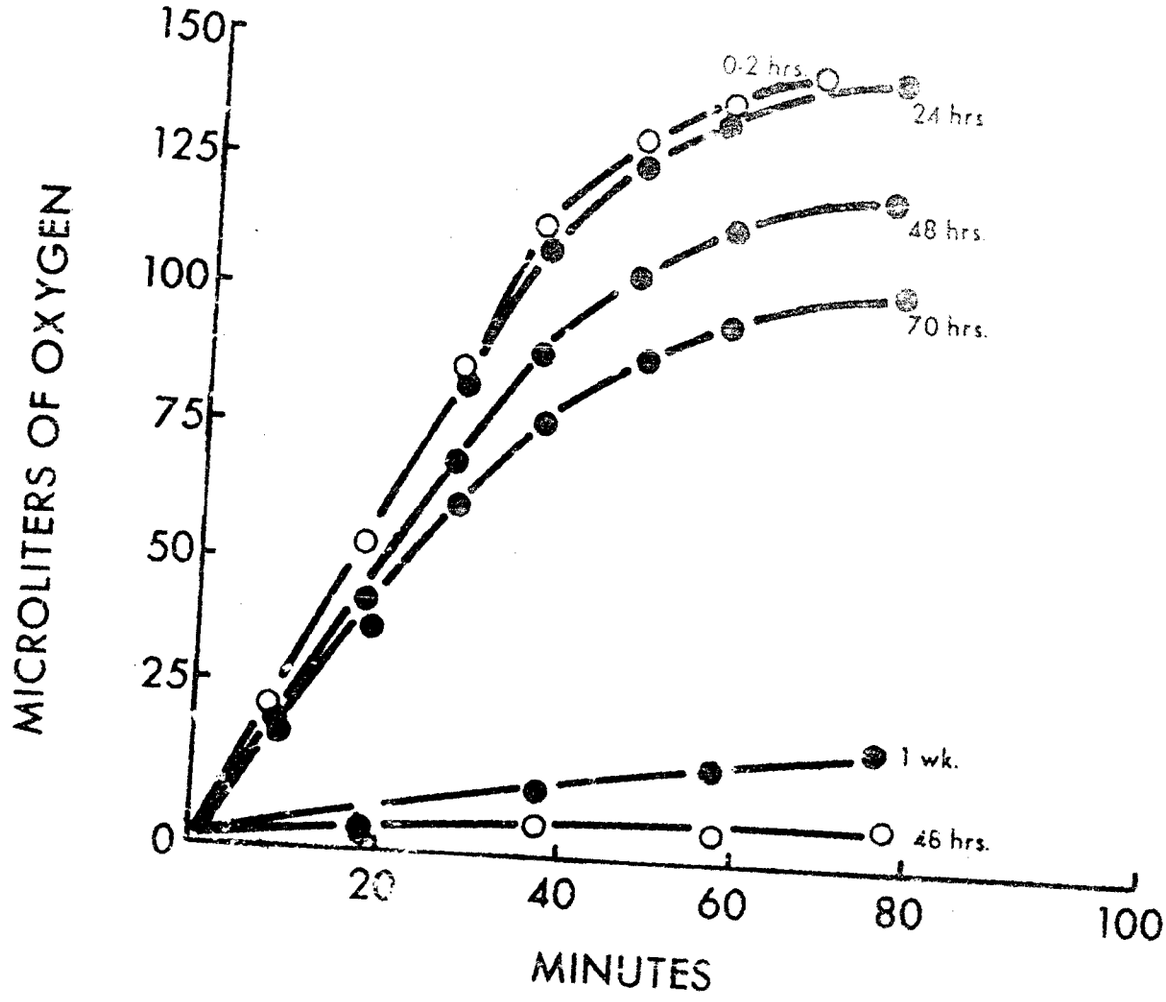



Figure 16. Effect of Storage on Glucose Oxidation by Heat Shocked Spores. The curves with the solid circles are for spores which were reheated for 60 minutes at 65°C.

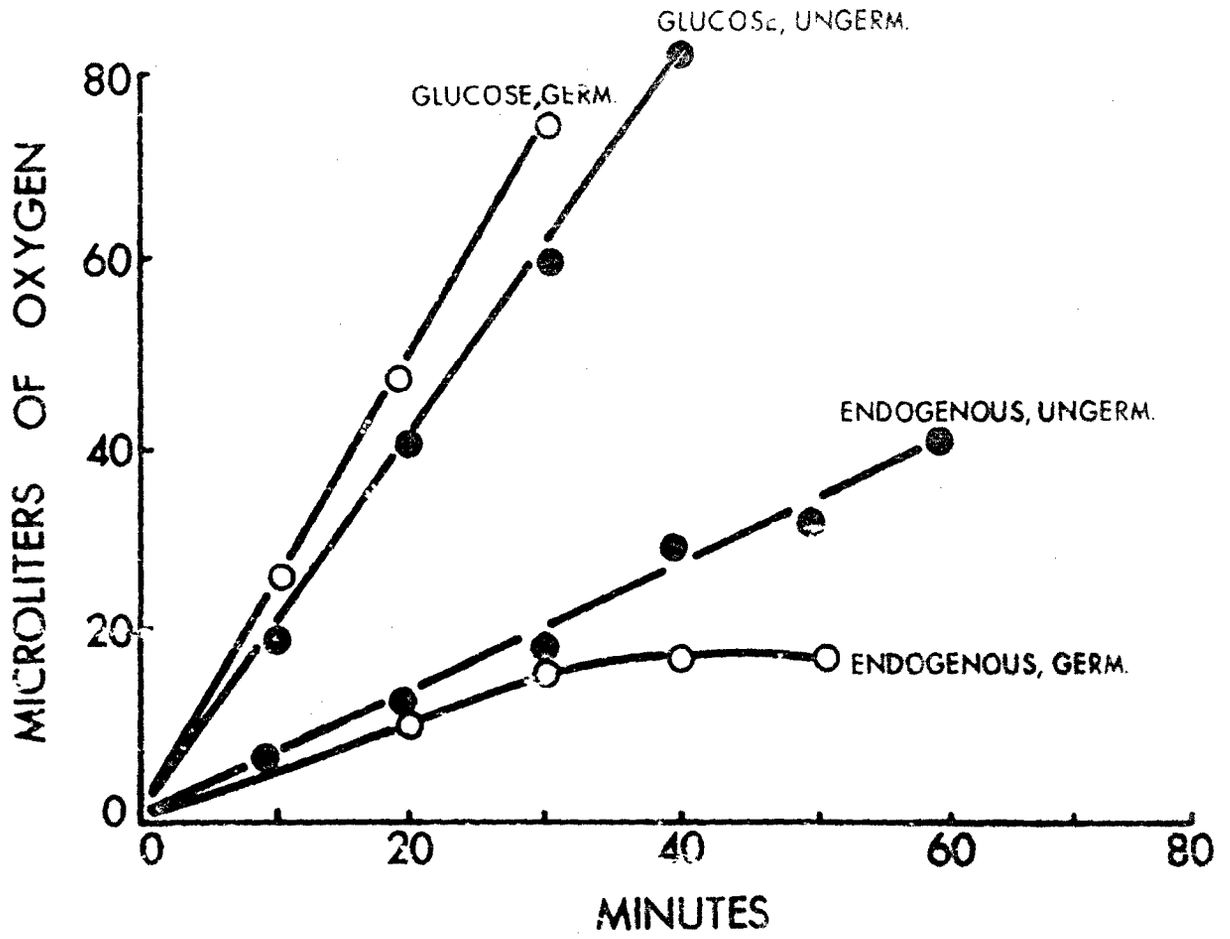
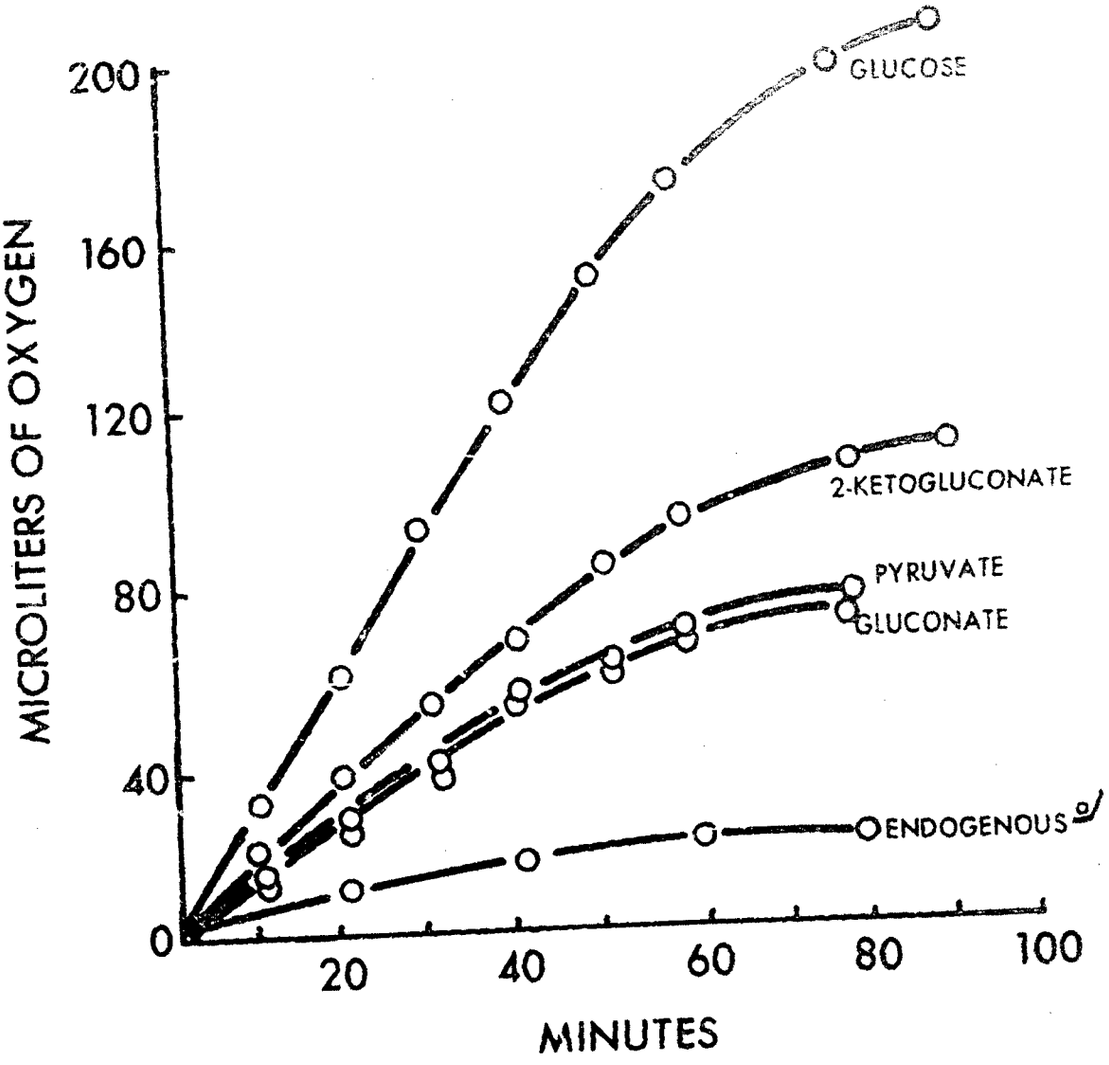



Figure 17. Activation of Respiration by Heat or Germination. The ungerminated spores were heated for 60 minutes at 85°C.




a. hexose diphosphate, glucose-6-phosphate, fructose-6-phosphate.

Figure 18. Carbohydrate Oxidation by Intact Spores of B terminalis.

We ruptured the spores by oscillation and by grinding in the Waring Blendor with superfine beads for a period of 30 minutes at 5°C (Figure 19). Rupturing spores is a very difficult procedure. We have not accomplished any rupture of 100 percent, but somewhere between 50 and 60 percent with the superfine beads in the Waring Blendor followed by centrifugation. This has been carried out in phosphate buffer. This bead extract is then placed in a Warburg flask in phosphate buffer and glucose added. The spore extract in the presence of yeast extract shows maximal oxidation. The yeast extract could be completely replaced by DPN. The spore extract then was dialyzed, and the dialyzed extract would also oxidize. These other materials were added to the dialyzed extract, indicating what components were necessary in this oxidation. Although we have been able to see that only the non-phosphorylating shunt is in operation in the intact spore, here is a requirement for ATP which would indicate that a phosphorylation is involved. We repeated that work which we did with the intact spore; that is, we used other carbohydrate substrates with the extract and could still obtain the activity with this material (Figure 20). With pyruvate we were still able to obtain significant oxidation. Glucinate and 2-keto glucinate, which were on the level with pyruvate in the intact spore, are negative here or of the order of the endogenous respiration.

SKIPPER: When you disintegrate your cells by sonic or other means, you then, I assume, spin down everything and just use the extract?

CHURCH: Yes, it is centrifuged at around 15,000g's in the Sorvall when you extract the spore. Figure 21 illustrates the pathway the glucose is travelling.

HALVORSON (Mich.): One thing that interests me very much is the fact that enzymes have been made active by means of heat shocking. The enzymes that have been active are heat resistant. The enzymes that have been exposed when the spore was ruptured and fixed in the supernatant are heat sensitive; but in germinating and rupturing the spore something has been done to the enzymes that make them lose their heat resistance. In the heat shocking alone, they are still heat resistant.

MANGUN: Well, they are the same enzymes, don't you think?

HALVORSON (Ill.): Yes.

MANGUN: I believe that. We only know of a few things that render enzymes heat stable; sodium caprylate, tryptophane, and related compounds will stabilize many of the proteins and enzymatic activity to plasma.

KAYE: I wonder if there is also the possibility of adsorption on smaller particles?

BEINERT: How about the enzyme's access to water? Proteins are more stable when they are dry. If you can find some way of denying the enzyme access to water, it will be more heat stable.

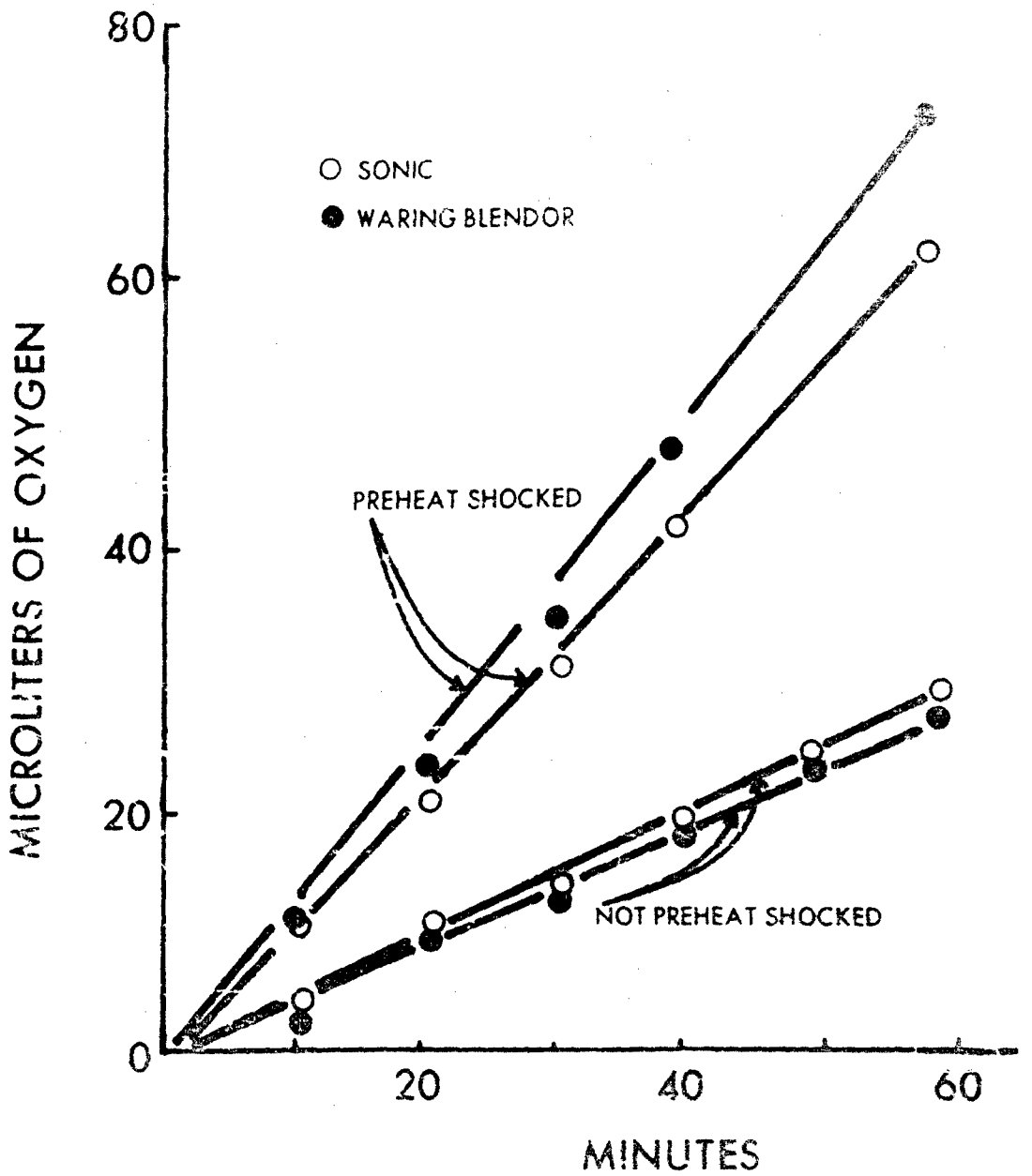
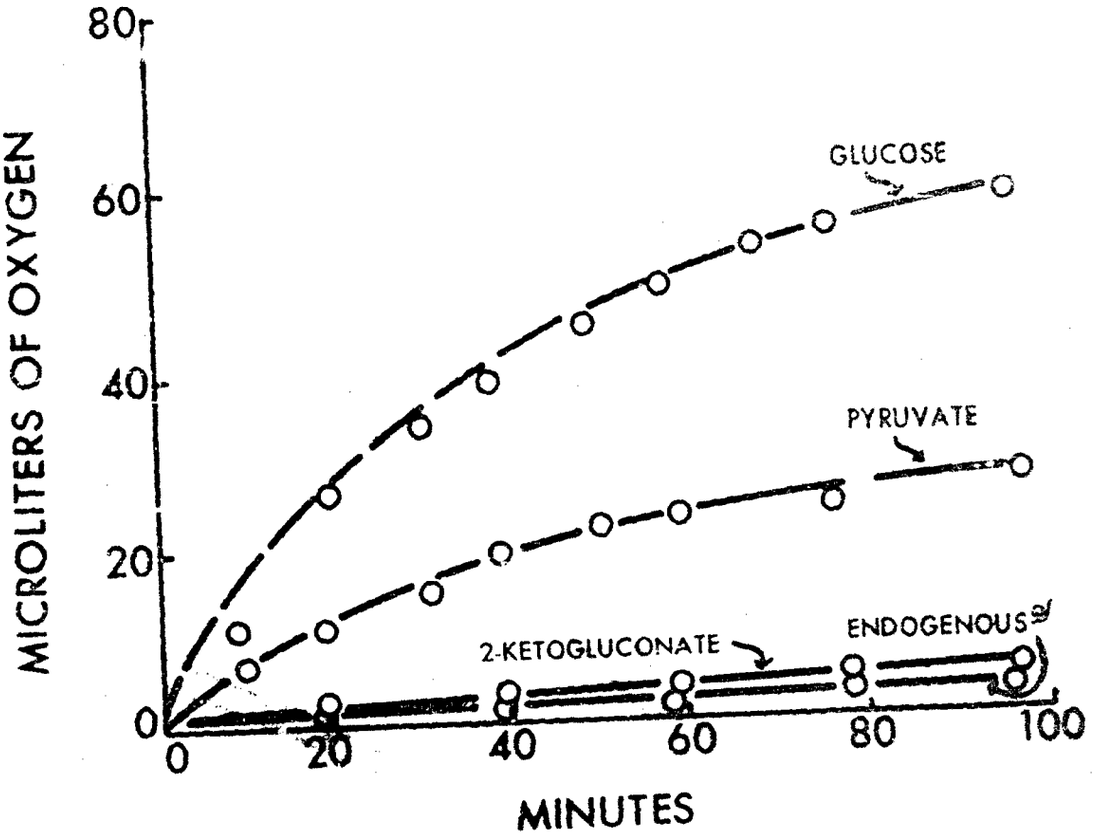



Figure 10. Effect of Preheat Shock on the Activity of Spore Extracts.




a. hexose diphosphate, glucose-6-phosphate, fructose-6-phosphate.

Figure 20. Carbohydrate Oxidation by Spore Extracts.

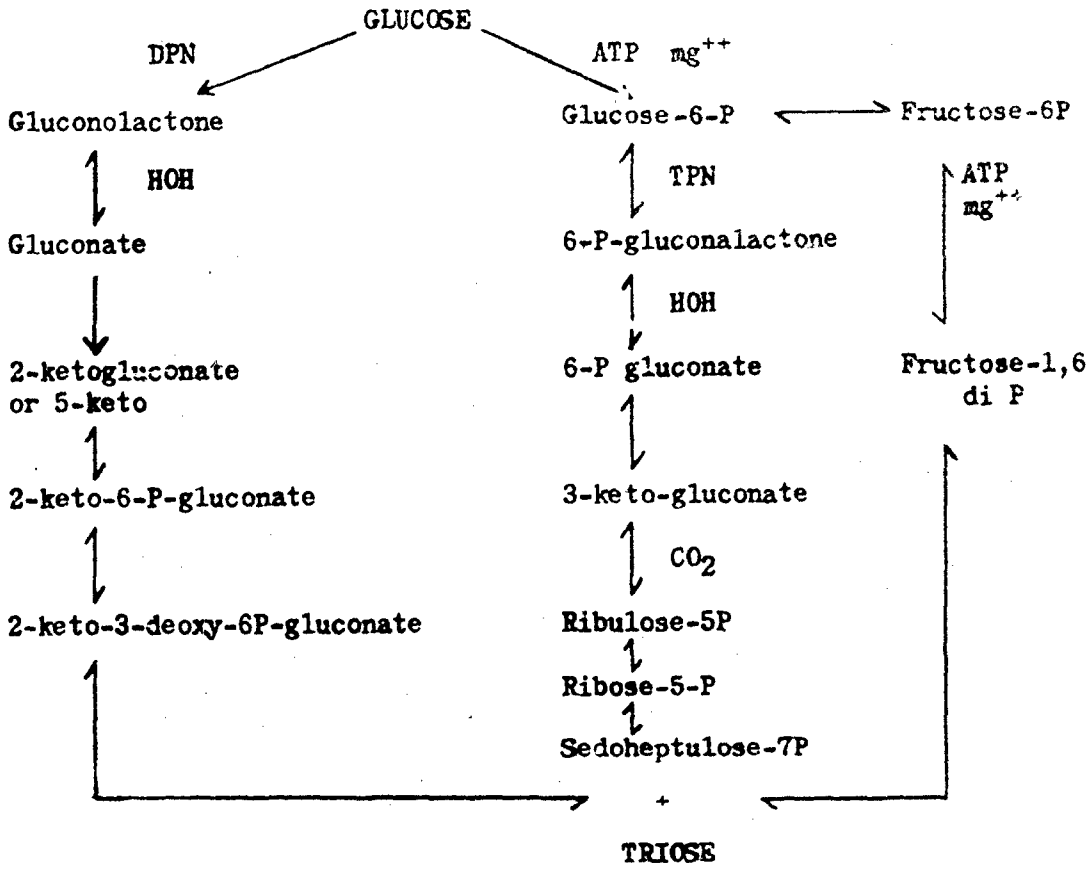



**Figure 21. Glucose Metabolism**

MANGUN: Well, since several inactivations are associated with protein primarily by bonding, one can certainly guess that the bonding is very effective in most cases. In essence, what substances like the caprylates do is tie things up so they can't whip around when they are subjected to thermal energy.

KAYE: How about dipicolinic acid that comes out of these spores? This is a very curious and interesting compound that may be associated here.

HALVORSON (Mich.): We have been very interested in that problem, and I am sorry that we didn't have more to say about it here. You have, of course, a tremendous amount of dipicolinic acid which spews out of the spore. It might be that dipicolinic acid, perhaps in a polymer form, confers heat resistance. We went after this problem to see if we could examine spores to find bound or free dipicolinic acid in heatshocked, unheatshocked, and germinated spores. The first problem was a methodological one. We spent most of our time getting methods for analysis of dipicolinic acid. We worked up a method based on the ferric chloride observation of Powell, and chromatographic separations. We don't have enough analytical data, but there is a fair amount of unpolymerized acid.

**WILLIAMS:** I am apologetic about starting in on our work because we don't have any explanations to offer. All I can do is give you a few little facts we have come across in comparatively recent times on the phenomenon which occurs in heat inactivation.

The general opinion about this very interesting phenomenon in connection with spores is that it is actually not heat inactivation but heat injury that takes place; that is, if a suspension of spores is exposed to a sublethal temperature, they are injured so that they cannot germinate or grow out as quickly as they could before. It takes a special media to recover them, and you get a little lower recovery. The question that this is an injury taking place is probably not a thing that ought to be criticized too much because the people who were working then were not doing anything calculated to show that it was otherwise. They were not doing the sort of experiment which is necessary in order to demonstrate heat activation, and these were more or less incidental observations.

Now what you need to do to show spore activation is to expose your suspension and observe the count before and after exposure. In 1943, Curran and Evans made their first observations on heat activation, and in 1950, Howard Griffith made a report on PA-833 where he obtained a considerable increase in the count as a result of exposure to heat. He got as much as a 12-fold activation with one of the organisms on the basis of 15 or 20 minutes of exposure. The observation that you can stimulate or accelerate germination by mild heating is fairly well established by now. Some years ago we tried to find out why and to accumulate some of the facts. I have only a few of these facts. and I'm not too sure about them. We have worked with five strains of thermophylls (three obligate and two facultative), with two strains of obligate anaerobes (Botulinum type A and PA 3639), and with seven strains of B subtilis, including two strains of var. niger, of differing heat resistance.

I might tell you about the preparation of the spores for testing and how it was done. The cultures on various media were run for about a week to ten days, the spores were washed three times with distilled water, and finally resuspended in very concentrated amounts in a 0.0333 M phosphate buffer from which they were diluted out in various suspending media. The next problem was to get rid of as many of the vegetative cells as possible without exposing to heat and without having inactivation. This was finally accomplished by sonic disintegration of the vegetative cells. When the suspension was exposed to ultrasonics, a constant count was obtained in 90 minutes. The vegetative cells were eliminated within 30 minutes, and even a run of an hour and a half did not decrease the spore count or alter the spore inactivation by heating. After the suspension was exposed to sonic treatment it was stored at low temperature until the experiments on the stock suspension were to be run. At that time, it was diluted out into pure media and exposed. After comparing a number of studies of recovery media, the one selected was brainheart starch reinforced media. We got higher counts with the brainheart starch media then with any other recovery media which we tried. There was actually only one strain out of this lot that gave any appreciable activation at all. The count was seven times as high after exposure to the temperature used for heat [illegible] as it was before.

If you activate spores and then store them, the activation will be lost fairly rapidly or comparatively so. It will be lost faster at a low storage temperature than it will be at a laboratory temperature. If you attempt to reactivate, you get very slight recurrence of the activation by taking these spores which were inactivated, stored, and reheated back again. The disappearance curve for activation goes down fairly rapidly. If you do not activate but do store the unheated spores, then they can be activated up to 17 or 18 months later. They do not die off very rapidly and they are still subject to activation. Let me repeat that. If you activate and store spores, the activation is lost and can be only partially restored by reheating. If you do not activate but do store spores, then they can be activated later.

One thing which has come out of this is that activation is not as common as we had once though it was. I think a person who is not familar with it would get the impression that activation can be induced or demonstrated with a spore suspension or any group of spores that you work with. Now our observation would indicate that this is not true, and while it can be found in some organisms, it will not stay there indefinitely. The organisms fluctuate in this respect. We also think it is important that skim milk seems the best medium to show this heat activation. The effect of milk is shown to correspond rather closely with the concentration of milk. If you dilute the milk by half with distilled water, you cut down the activation from a six-fold increase, let us say, to maybe about four. If you cut it in half again, you cut it down to between two and three. If you get as low as 10 percent milk, you get back in the range of distilled water. Exactly what is concerned here, we do not know.

MANGUN: Will milk ash have any effect in this connection?

WILLIAMS: That was tested. The milk ash does not have any significant effect.

HALVORSON (Mich.): Before you heat your suspension, how does a direct count compare with a viable count?

WILLIAMS: Well, unfortunately, I do not have the data on direct count. That was done; but I don't have the data with me, and I can't tell you.

HALVORSON (Mich.): I am just a little surprised that you get an increase in plate count by heating. Whether it is a function of your beef heart infusion broth, I don't know; but I know with the spores we have looked at, and we have looked at seven now, we get 100 percent of our spores to grow in the plate unless they are very old. If they are very old, the viability begins to decrease. But with fresh spores, we get 100 percent without heating, comparing direct counts with viable counts.

WILLIAMS: No, that wasn't the case in our work. With this organism, that was true; you got an increase in count as a result of heating, a tremendous increase in count in some instances.

LEONARDS: I have been asked to say a little about our work on spores, which is really of a different nature. Our work deals exclusively with the effect of beta-propiolactone in the vapor state and it has but two virtues: first, the technique is extremely simple, and second, my presentation is extremely short. We have a big box, with a volume of 1300 liters, into which we atomize propiolactone after proper adjustment of the humidity. Then we analyze the propiolactone in the air by withdrawing one-liter samples of air and treating it with hydroximine and ferric chloride in the usual manner. In that way, we can follow the destruction of lactone, which as you know, is an unstable compound even in the vapor state. By varying the concentration of lactone, by varying the humidity, and by taking samples of spores out of the chamber at various time intervals, we hope to be able to get some idea about just exactly what conditions have to exist in order to get spore destruction under practical conditions. Figures 22 and 23 are merely indications of the relative instability of propiolactone, and obviously, the instability is greater as you increase the humidity. The two figures represent lactone at two different initial concentrations: a high concentration of about 9 milligrams per liter and a low concentration of around 2½ milligrams per liter. Note that, after just a few minutes in the chamber at the highest humidity (22.9 milliliters of mercury water vapor), immediate sampling reduced the concentration of lactone in the chamber from about 8.8 to 5.5 milligrams per liter (Figure 22). In other words, at a high humidity, the material is quite unstable.

SKIPPER: What is its vapor pressure at 25°C?

LEONARDS: Between 2.6 to 2.8 millimeters of mercury, I believe, and that is equivalent to a maximum obtainable concentration of 10.5 milligrams per liter. The maximum obtainable concentration in the big chamber that we have was only 9.5 milligrams per liter by actual analysis, and even that is difficult to obtain. Figure 24 (we have only done a few of the conditions) illustrates two things. One is that even the highest concentration of lactone that is obtainable (9.5 mg/l) is almost completely ineffective against B subtilis var. niger spores. I should mention that the spores are impregnated in little cloth patches about the size of a quarter, and we count the number of spores by shaking the patches in 100 cubic centimeters of distilled water and then do plate counts. At humidities of a little less than half-saturation (11.5 mm Hg $H_2O$), the compound is somewhat effective. If you are thinking about absolute sterilization, you may as well forget about it; the count is reduced by about only one log, from $10^7$ to a little above $10^6$. Incidentally, I'm sure the rise indicated on Figure 24 isn't real. I might mention that about the best we can do is to extract 30 percent of the spores which we put on the patches when we try to recover by shaking.

HALVORSON (Mich.): Did you drop your patch into a growth medium?

LEONARDS: Well, we can drop them into a growth medium, but we have to count them. Essentially, we do drop them into a growth medium if we want to check for sterility.

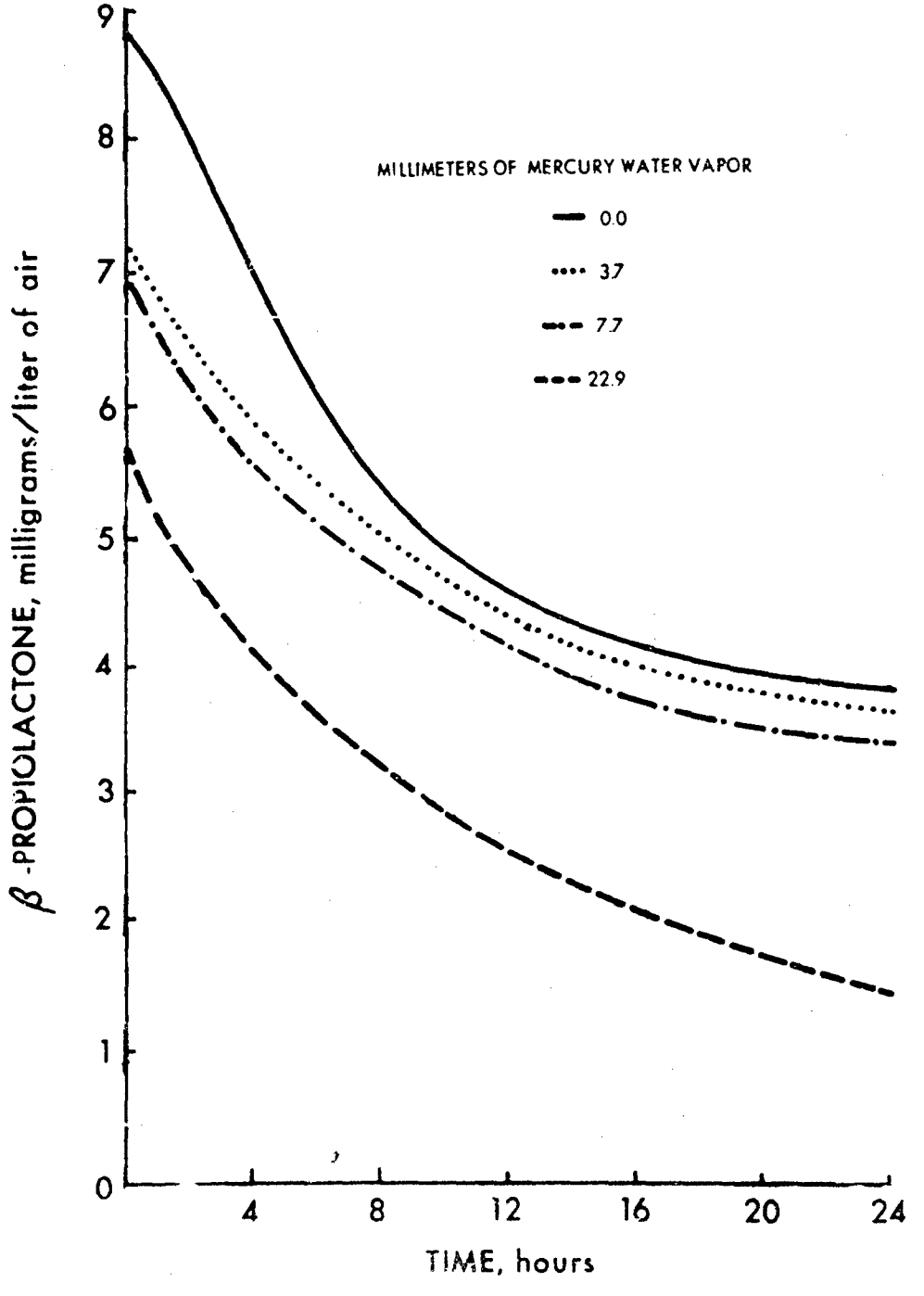



Figure 22. The Effect of Humidity on the Stability of Beta-propiolactone in the Vapor State. The initial concentration of beta-propiolactone was about 9 milligrams per liter.

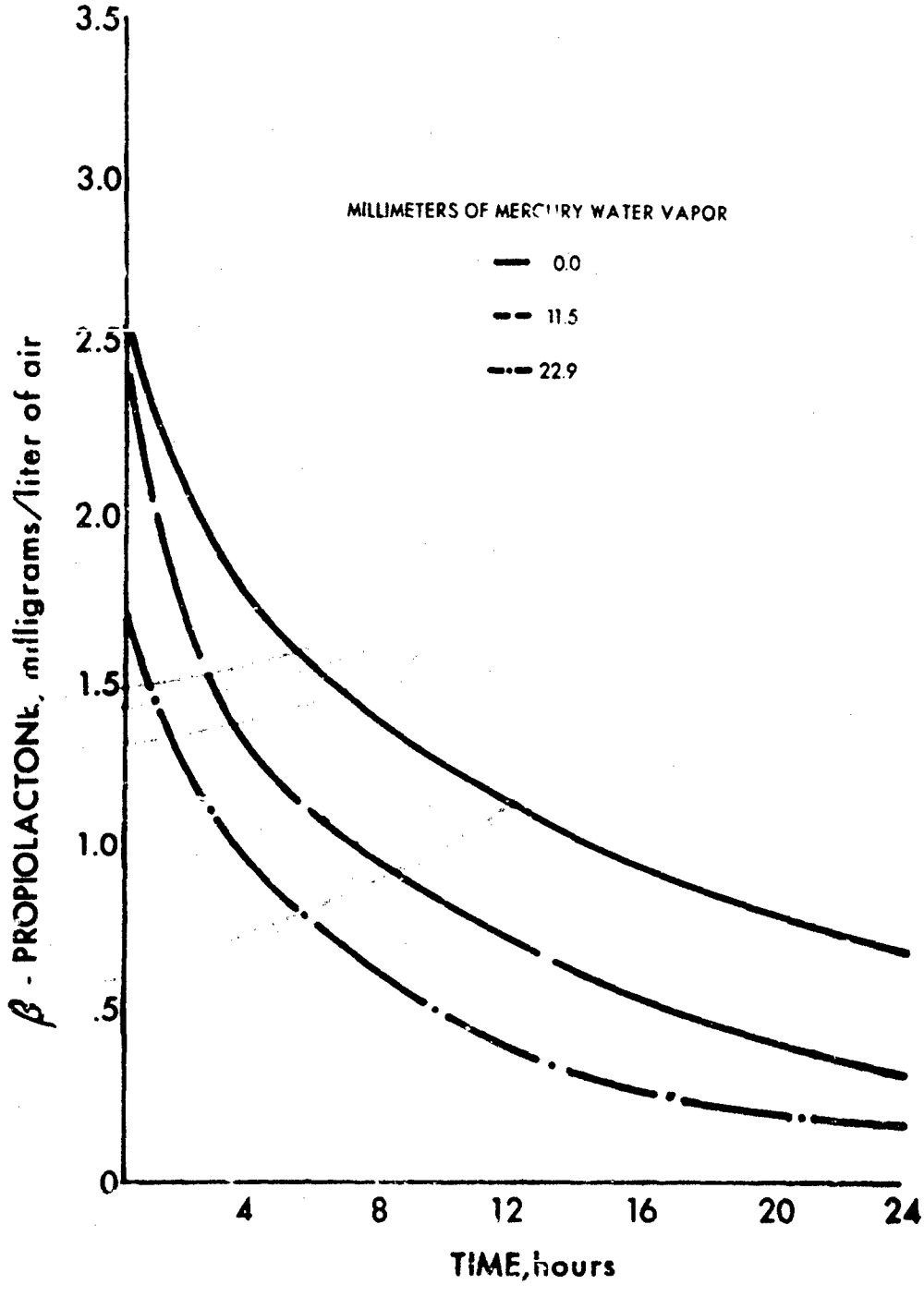



Figure 23. The Effect of Humidity on the Stability of Beta-propiolactone in the Vapor State. The initial concentration of beta-propiolactone was about 2½ milligrams per liter.

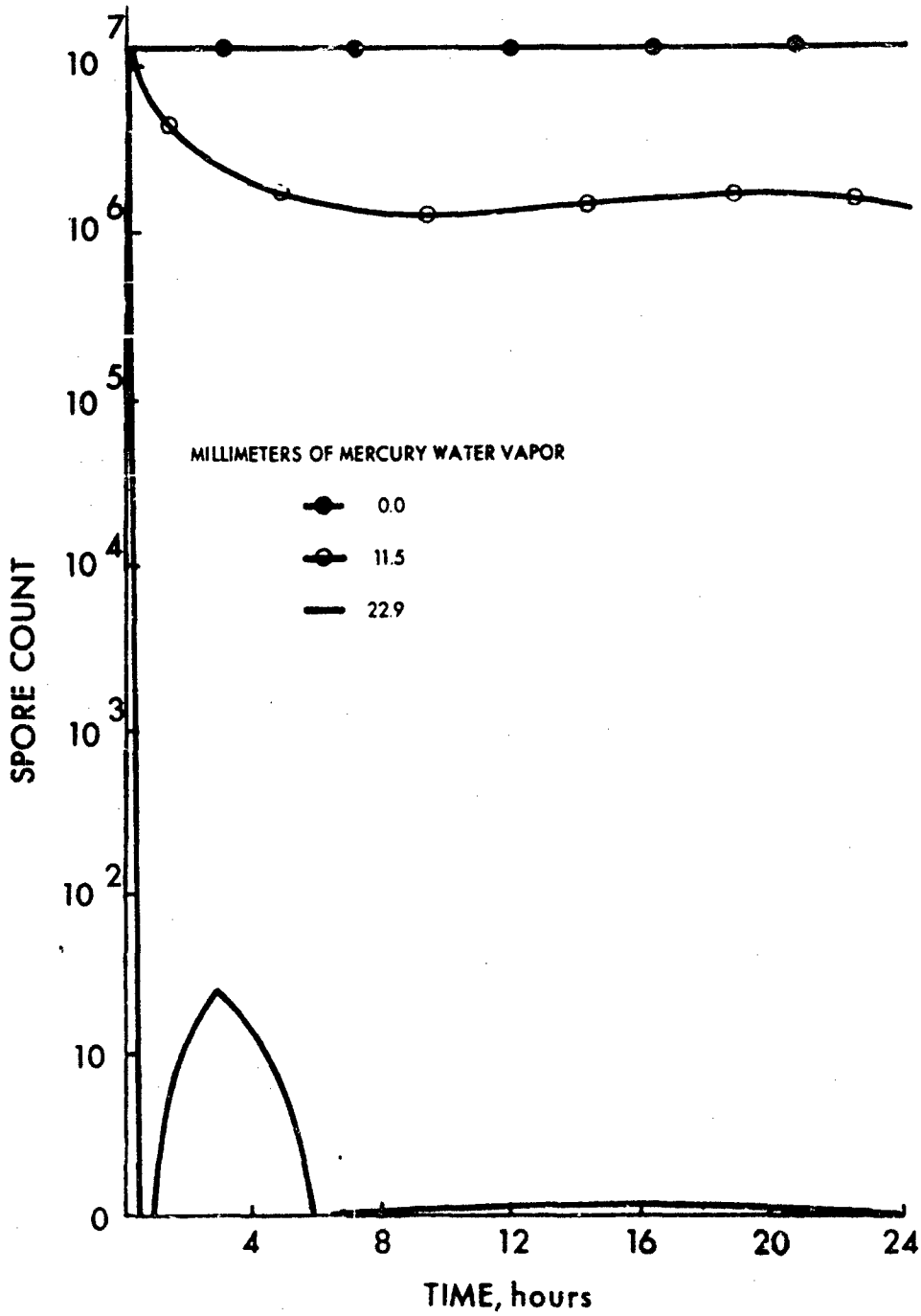



Figure 24. The Effect of Humidity on the Efficacy of Beta-propiolactone on B subtilis var. niger Spores. The initial concentration of beta-propiolactone was about 9.5 milligrams per liter.

CHURCH: There was a study done at Chicago in which the patches impregnated with organisms were ground in Waring Blendors, and 100 percent recovery was obtained that time.

LEONARDS: Well, that might be an improvement over the technique. I might mention that because this is a completely practical aspect of the work, we are one of the few contractors that have our procedures written out for us to begin with. This washing technique has been used at Detrick for many years, and they decided to keep that standard so they can compare it with previous results.

SKIPPER: If you put up your close-to-saturation concentration at 25°C in your chamber, how rapidly does that concentration fall off?

LEONARDS: Well, I think that is on Figures 22 and 23. On both these charts, the top curve is for zero millimeters of mercury and the bottom curve is what you get with high humidity.

SKIPPER: There is no air going in or out?

LEONARDS: That is right.

SKIPPER: It falls off just by adsorption on the walls.

LEONARDS: By adsorption on the walls, and probably also by destruction of the lactone, which is known to be unstable in the presence of moisture.

MANGUN: In the vapor phase?

LEONARDS: In the vapor phase.

MANGUN: Has anyone seen this type of thing in an aqueous media where you knock out almost all the spores, but there are a few that don't get knocked out?

LEONARDS: Well, we are not hitting the spores, we are hitting cloth patches in which spores are impregnated.

MANGUN: I know. What I want to bring out is a possible explanation of this thing based on some observations made during the war with gas chambers. A rabbit was put in a gas chamber along with quite a variety of chemicals, of which lewisite was the most remarkable. Chemical samplers were placed in the middle of the chamber. Let's say a certain nominal concentration was desired, and 30 to 90 percent optimal concentration was obtained by direct analysis. If the sampler was placed two inches from the fur of the rabbit, however, the concentration was almost zero. There must be something like that going on here. It is an interesting practical point; perhaps this does happen.

KAYE: I think we have to exercize caution here and avoid conclusions based on the bacteriological counts that have been made thus far. This is the sort of test that is set up so that you have to wait until all the data are collected before you know what the first experiment did.

LEONARDS: I think that the most important practical aspect of this thing is that, in the vapor state, propiolactone is very ineffective unless the humidity is very high; even at half-saturation at 11.5 millimeters mercury, the largest concentration of lactone obtainable in the vapor state is relatively ineffective. I think this is the main conclusion that we can reach: if beta-propiolactone is to be of practical use in the vapor state, it just has to be wet.

CHURCH: I don't think Figure 24 indicates what effect the high humidity had on your organisms.

LEONARDS: Oh, it has none. I don't know what effect it has on the resistance or the organism, but it has none on the count. Is that what you are thinking about?

CHURCH: I was wondering if the organisms were active at low humidity.

LEONARDS: Well, we can take the organism and dry it on the patches, keep them over concentrated sulfuric acid for three months, then extract them, and still get the same count as if we kept them wet.

SHAVER: I'll throw this in for what it is worth: The Department of Agriculture reported some work done on the treatment of wool with lactone to improve felting properties. Lactone reacted more actively with wool at a higher humidity. Perhaps there is a similarity between the protein in your organism and wool. At one time, I thought this was a swelling effect, which meant that penetration was getting into the active site. That is all we know about it.

KAYE: The problem of neutralizing organisms has plagued everybody over all these years, yet there isn't very much data, I'm afraid, on whether in vitro neutralization that might be done in the agar corresponds with in vivo neutralization in the body.

ENGLEY: I have been asked to comment on the reactivation, or reincarnation of, or revitalization of these "mercurial-killed" organisms. The mercurial compounds present a very interesting group of chemicals. It's been known since at least 1890 that the mercurial's activity as a disinfectant or antiseptic could be neutralized with sulfhydryl-containing chemicals, so today I have a few charts and tables borrowed from other people, some work of our own, and a few comments on work that we have going on at the present time.

Now, the technique that we have utilized for neutralization is the actual injection of the chemical-organism mixture into a susceptible animal after exposure, doing this at the same time with nutrient broth and thioglycollate-containing media. This technique of just putting the organism into the animal isn't new with us. Dr. Nungester at Michigan worked up a technique of actually putting the organisms on a mouse's tail, exposing to the disinfectant, snipping off the tail and putting it inside the mouse. One can't do many series of compounds or too many dilutions at any particular time, but the technique is very interesting. He used both streptococcus and pneumococcus for that particular test, as shown in Table XII.

Pierce and Tilden did the same work with different compounds (Table XIII). Early in the '40's, we carried out similar work by injecting intraperitoneally the material containing the chemical plus the organisms. Table XIV contains data from our later studies. I'd like to point out here the fact that it correlates directly with the test tube inactivation with thioglycollate or with serum. Now there are all sorts of inhibitors and neutralizers for chemicals in the test tube. But the important point here is do these chemicals run into this type of inhibitor under practical use conditions? Here we have a practical use condition, where serum contains sulfhydryl groups as does the body. I think that this type of test might be continued with other groups of organisms where we have no inhibitors. This type of test could be used practically by exposing the organisms to the compounds, and injecting the chemicals and the organisms into the body to see if those organisms have been killed or inactivated to the point that they no longer can cause infection.

In Table XV, we have indicated a test wherein we used the paper disc that has been used for antibiotic assay. We can compare the diameter of the zone of inhibition caused by the various compounds on nutrient agar; then, if we put thioglycollate into the medium, there is no longer any inhibition of the organism. If we carry it out more practically by putting in 50 percent serum (and actually blood does contain about 50 percent serum), then we find that these chemicals have no inhibiting power.

Now this same paper disc assay technique has an advantage in the testing of this type of compound where it is applied as a tincture; this technique (Table XVI) will give the same size zone for the aqueous preparation as the tincture so that we can get rid of the solvent effect in mixtures of chemicals This is very nice, because in doing a phenol coefficient on a tincture preparation, the activity of the mixture often is due to the alcohol or acetone in the mixture.

Yesterday, there were some comments about the use of two chemicals together with detergents present. Table XVII shows some similar data. If the surface-active agent is cationic, it has strong activity on the cells, and you will get good zones sometimes with the serum agar present. Most of the surface-active agents are fairly well neutralized in the presence of large amounts of organic matter. In this particular technique we were using Staphylococcus aureus.

TABLE XII. RESULTS OF IN VIVO TESTING OF DISINFECTANTS[a]

| DISINFECTANT | | ORGANISM | ANIMALS INOC. | % DEAD |
|---|---|---|---|---|
| Tinc. Iodine | 2.0% | Streptococcus | 29 | 48 |
| Tinc. Mercresin | 0.1% | | 31 | 94 |
| Tinc. Merthiolate | 0.1% | | 27 | 96 |
| Tinc. Phemerol | 0.1% | | 31 | 90 |
| Tinc. Iodine | 2.0% | Pneumococcus | 15 | 0 |
| Tinc. Mercresin | 0.1% | | 48 | 23 |
| Tinc. Merthiolate | 0.1% | | 40 | 55 |
| Tinc. Phemerol | 0.1% | | 52 | 67 |
| Tinc. Phemerol | 0.2% | | 56 | 16 |
| Tinc. Vehicle | | | 47 | 68 |

a. Nungester and Kempf, J Infec Dis, 71:174, 1942.

TABLE XIII. RESULTS OF IN VIVO TESTING OF DISINFECTANTS

| DISINFECTANT | | ORGANISM | ANIMALS INOC. | % DEAD |
|---|---|---|---|---|
| Tinc. "D.C.-12" | 0.1% | Pneumococcus[a] | 120 | 42 |
| Tinc. Metaphen | 0.1% | | [illegible] | 85 |
| Tinc. Merthiolate | | | [illegible]4 | 97 |
| NaCl | 0.85% | | [illegible] | 98 |
| Mercurochrome | 2.0% | Streptococcus[b] | 8 | 63 |
| Metaphen | 0.2% | | 8 | 25 |
| Merthiolate | 0.1% | | 6 | 66 |
| Phenol | 1.0% | | 4 | 0 |

a. Pierce and Tilden, J Dent Res, 24:5, Oct. 1945.
b. Morton, et al, J A M A, 136:37, Jan. 3, 1948.

TABLE XIV. COMPARISON OF IN VIVO AND IN VITRO METHODS OF EVALUATING MERCURIAL ANTISEPTIC ACTIVITY

| COMPOUND | CONC. OF USE, Percent | MICE DEAD / MICE INJECTED[a] | DEXTROSE BROTH[b] ALONE | + 0.1% THIOGLYCOLLATE | + 10% SERUM |
|---|---|---|---|---|---|
| Phenyl mercuric borate | 0.1 | 8/10 | - | + | + |
| Merbak | 0.1 | 9/10 | - | + | + |
| Merthiolate | 0.1 | 10/10 | - | + | + |
| Mercurophen | 0.1 | 9/10 | - | + | + |
| Mercuric iodide | 0.1 | 8/10 | - | + | + |
| Mercarbolide | 0.1 | 8/10 | - | + | + |
| Merodicein | 0.2 | 10/10 | - | + | + |
| Metaphen | 0.2 | 7/10 | - | + | + |
| Mercurochrome | 2.0 | 10/10 | - | + | + |
| Mercuric chloride | 0.1 | 10/10 | - | + | + |
| Water | | 10/10 | + | + | + |

a. Mice injected and tubes inoculated with 0.1 milliliter of a 1:2 dilution of Streptococcus — mercurial mixture after a ten minute exposure at 32° to 34°C. Streptoccocus recovered from heart's blood at autopsy.

b. - = no growth within 7 days; + = growth of test organism.

TABLE XV. COMPARISON OF THE EFFECT OF NEUTRALIZERS ON INHIBITION ZONES BY THE PAPER DISC ASSAY METHOD

| COMPOUND | DILUTION OF USE, Percent | NUTRIENT AGAR INHIBITION ZONE,[a] millimicrons | NUTRIENT AGAR + 0.2% THIOGLYCOLLATE | 50% SERUM AGAR |
|---|---|---|---|---|
| Phenyl mercuric borate | 0.1 | 33 | - | - |
| Mercresin | 0.1 | 32 | - | - |
| Mercarbolide | 0.1 | 32 | - | - |
| Merthiolate | 0.1 | 32 | - | - |
| Mercurophen | 0.1 | 29 | - | - |
| Mercuric Iodide | 0.1 | 28 | - | - |
| Metaphen | 0.2 | 25 | - | - |
| Mercurochrome | 2.0 | 24 | - | - |
| Merbak | 0.1 | 23 | - | - |
| Merodicein | 0.2 | 18 | - | - |
| Mercuric chloride | 0.1 | 18 | - | - |

a. Twenty-milliliter plates were used.

TABLE XVI. COMPARISON OF AQUEOUS AND TINCTURE MERCURIALS BY THE PAPER DISC ASSAY METHOD

| PREPARATION | INHIBITION ZONE,[a] millimeters |
|---|---|
| Merthiolate, Aqueous | 30 |
| Merthiolate, Tincture | 29.5 |
| Merbak, Aqueous | 22 |
| Merbak, Tincture | 22 |
| **CONTROLS** | |
| Alcohol, 95% | — |
| Alcohol, 50%; Acetone, 10% | — |
| Water | — |

a. Twenty-milliliter plates were used.

TABLE XVII. COMPARISON OF THE EFFECT OF SURFACE ACTIVE AGENTS ON MERCURIAL ACTIVITY BY THE PAPER DISC ASSAY METHOD

| MERCURIAL | SURFACE ACTIVE AGENT | MEDIUM | | |
|---|---|---|---|---|
| | | NUTRIENT AGAR | NUTRIENT AGAR + 1% THIOGLYCOLLATE | 50% SERUM AGAR |
| | | Zone of inhibition, millimeters [a] | | |
| Mercurochrome 1% | | 23 | — | — |
| Mercurochrome 1% | Plus Duponol 1% | 25 | 26 | — |
| | Duponol 1% | 24 | 23 | — |
| Mercurochrome 1% | Plus Roccal 1% | 20 | 17 | 16 |
| | Roccal 1% | 26 | 25 | 18 |
| Mercurochrome 1% | Plus Triton A20 1% | 27 | — | — |
| | Triton A20 1% | — | — | — |

a. Five-milliliter plates were used.

I think it is very important in testing chemicals, not only to do it in the test tube, but also to do some use applications to see if they are going to work in practice, whether it is in stainless steel pans, on the skin, or on impregnated cloth.

Another important point on the mercurials we've been studying in the past few years. Mercurials, you know, are used very frequently as preservatives in vaccine and in serum, and I've always worried about them because the mercurial compound will get tied up with protein very readily. We set up typhoid vaccine, added mercurials and other preservatives to the vaccine in the refrigerator, and then added a standard number of organisms: 300 per milliliter of B subtilis spores, S aureus cells, and Escherichia freundii L (because that's a common contaminant of plasma). If we add the organisms at zero time when we add the chemicals, we find that the mercurials are very effective in controlling the organisms that are added; however, if we let that vaccine sit for six months in the refrigerator with the chemicals present and then add the organisms, we find that, in the case of the mercurials in particular, the organisms stay alive. As a control on this, we have used formaldehyde, and find that the formaldehyde is still able to prevent these organisms from growing after six months. In plasma, these mercurial compounds have even less activity. We haven't done this quantitatively to find out how much of the mercurial is taken up as we increase the amount of protein.

Now it has been shown in previous work that spores remained viable in mercurial preparation for as long as ten years, and when taken out and put into thioglycollate medium, or into serum medium, they became reactivated, germinated, and grew into colonies. This brings up the question of the use of mercurials as preservatives, and it also brings up another problem that we are just running into. If we have a lot of protein present in the vaccine and mercurial is added, then it doesn't do any particular inactivation of the vaccine antigen. However, in the polio vaccine which is being put out on the market, they ran into difficulties with preservatives in this respect: if they added merthiolate alone to the polio vaccine, the merthiolate not only combined with the polio virus but destroyed it as an antigen. Now they have added versene to the polio vaccine to control the merthiolate. We've just started tests to determine whether the mercurial is going to be a preservative when it is tied up by the versene. So you can see the difficulty that we run into with the mercurials.

I think that practical types of tests such as the ones that we've used should be applied to other chemicals in order to answer the question of whether the organisms are killed, inactivated, or so devitalized that they can no longer cause infection in the experimental animals which are susceptible. I think that should be kept in mind with the testing of the other chemicals. Some of the inactivators or neutralizers that are used have no relation to actual body conditions, although they are very interesting to use in the laboratory to see if you can get the organisms back.

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